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ACTION OF PLANT GROWTH REGULATORS III. ADSORPTION OF AROMATIC ACIDS TO OAT MONOLAYERS[1]

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In an earlier paper (2) it was concluded that a broad correlation exists between the resistance of a plant to 2-methyl-4-chlorophenoxyacetic acid (MCPA) and its ability to adsorb the growth regulator. This lent support to the suggestion that the foundation of species resistance may be partly based on the extent of MCPA adsorption to sites not concerned in the physiological response.

This work has been extended since it follows that the biological activity of different growth regulator molecules on a given plant species may also be limited by the extent of their adsorption, and a correlation may exist between biological activity and the adsorption of growth regulators to plant constituents. This has already been implied by Veldstra and Booij (9) when noting that chlorination of the phenoxy compounds generally appeared to increase their activity but chlorination of naphthoxy compounds reduced their activity. Similarly, these authors consider that certain trichlorophenoxy acetic acids have exceeded the optimum size and hence optimum interaction with plant membranes, for high biological activity.

Three series of acids were investigated. The phenoxyacetic and benzoic acids were selected in view of the importance of some members of these series as herbicides. The phenylacetic acids were studied as a result of earlier observations at Jealott's Hill in 1953 that 2,3-dichloro and 2,6-dichlorophenylacetic acids were compounds with biological activity similar in character but somewhat inferior to MCPA.

This paper presents physical measurements of the adsorption of the acids to monolayers from the squashes prepared from oat leaves using the Langmuir trough. No biological data are presented in this paper but it is hoped it will be possible to seek a correlation when the results of biological tests on phenylacetic acids being made by various research teams become available. It is proposed to present these in Part IV of this series.

[1] Received manuscript November 3, 1959.

MATERIALS AND METHODS

APPARATUS. A Langmuir trough and surface balance accurate to 0.2 dynes/cm were used to study the adsorption of the acids to monolayers of components from oat leaves as previously described (2, 3). The principles involved and the method of studying monomolecular layers are fully described in a textbook by Adam (1).

By this technique, a surface layer one molecule in thickness from oat leaf squashes was studied. Force-area (F-A) curves were an indication of the cohesive forces between molecules in the surface and the degree to which the injected organic acid had penetrated. Surface potential-area (Δ V-A) curves enabled the degree of interaction between the surface molecules and the injected acid to be assessed.

Surface potentials (Δ V) were determined by means of a radioactive electrode in circuit with a triode valve electrode.

EXPERIMENTAL

The preparation of the films was identical to that described in earlier papers (2, 3). It was not possible to assess the weight of material spread and hence standard conditions were maintained throughout the work by spreading until a pressure of 3 dynes/cm was built up. As a result, the usual units (F-A) and (Δ V-A) could not be used and instead (F-l) and (Δ V-l) curves were constructed, l being the distance between the mica float of the surface balance and the moveable barrier. l is proportional to the area of film on the trough.

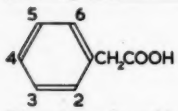
As previously described, monolayer interactions of the acids were studied by plotting (F-l) and (ΔV-l) curves for monolayers on a pure substrate and comparing them with similar monolayers in equilibrium with a substrate containing the acids. Adsorption of the injected acid was indicated by a change in surface potential

(i.e.) Δ V film $-$ Δ V film/acid $= \delta \Delta$ V

and/or an increase in surface pressure.

TABLE I

MELTING POINTS AND IDENTIFICATION CODE OF α-INDOLE AND α-NAPHTHYLACETIC ACIDS AND PHENYLACETIC ACIDS

COMPOUND	ABBREVIATION	MELTING PT° C
		
Phenyl acetic acid	PA	79
Indole acetic acid	IA	
α–Naphthyl acetic acid	α-NA	132
2–Chlorophenyl acetic acid	2CPA	94
3–Chlorophenyl acetic acid	3CPA	80
4–Chlorophenyl acetic acid	4CPA	104
23–Dichlorophenyl acetic acid	23DCPA	131
24–Dichlorophenyl acetic acid	24DCPA	131
25–Dichlorophenyl acetic acid	25DCPA	106
26–Dichlorophenyl acetic acid	26DCPA	159
34–Dichlorophenyl acetic acid	34DCPA	88
35–Dichlorophenyl acetic acid	35DCPA	113
234–Trichlorophenyl acetic acid	234TCPA	178
235–Trichlorophenyl acetic acid	235TCPA	144
245–Trichlorophenyl acetic acid	245TCPA	124
236–Trichlorophenyl acetic acid	236TCPA	158
246–Trichlorophenyl acetic acid	246TCPA	218
345–Trichlorophenyl acetic acid	345TCPA	146
34–Dimethoxyphenyl acetic acid	34DMOPA	82

The growth regulator was injected in aqueous solution beneath the monolayer already at equilibrium on the surface. Gentle but prolonged stirring ensured that the substrate was homogeneous. Acetate buffers of pH 4.30, 4.70 (ionic strength I = 0.005) were used as substrates.

All standard reagents were of "Analar" quality. The organic acids used were mainly prepared by Dr. R. L. Jones[2], synthesised by methods designed to eliminate extraneous isomers and purified by several crystallisations. The acids, their abbreviated names and melting points are given below in tables I, II and III.

COMPUTATION OF RESULTS. Unlike the work reported in Part II of the series (3), the extent of adsorption was determined by assessing the percentage of adsorbing surface covered by a given concentration of injected acid. It was obtained from a Langmuir isotherm (2). This procedure was necessary since surface potential changes resulting from the injection of a series of acids arise from two factors: A, the dipole of the injected acid, and B, the amount of acid adsorbed. By assessing adsorption from the proportion of surface covered, structural contributions were eliminated.

Thus $\delta \Delta V$ was determined at a concentration of 41×10^{-5} M and compared with that at surface saturation. Adsorption is expressed as percentage surface covered at concentration 41×10^{-5} M (see fig 1). Surface saturation values could not be found by direct experiment and were obtained by plotting $c/\delta \Delta V$ versus C (fig 1). The inverse slope of this line gave the value $\delta \Delta V_s$ (2). No assumptions relating to film reorientations are involved in this method but it has the disadvantage that it gives no absolute measure—merely the surface affinity for the injected acid. As, however, we wish only to assess the relative adsorptions of a series of acids, this method is considered entirely adequate.

Figure 1 gives the adsorption isotherm for 345-trichlorophenyl acetic acid at 20° C. Surface covered in percentage was assessed at 41×10^{-5} M acid because this point lies at the top of the region where surface coverage increases rapidly with increasing concentration of injected acid. Thereafter, the curve flattens and much larger quantities must be injected to affect materially the amount of 345TCPA adsorbed.

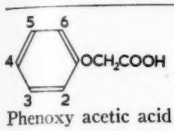
Experiments were carried out at pH 4.30 and pH 4.70. This was done to ensure that differences in the interactions of the acids were due to structural variation and not merely to differences in the proportion of ionic form present at the selected pH.

The films were similar to those of proteins or lipoproteins and exhibited elastic properties as shown by the recoil of talc placed on the film-covered surface

[2] I.C.I. Ltd., Pharmaceuticals Division, Alderley Park.

TABLE II

MELTING POINTS AND IDENTIFICATION CODE OF PHENOXY, α-AND β-NAPHTHOXYACETIC ACIDS

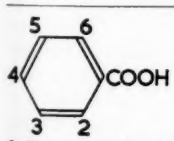
COMPOUND	ABBREVIATION	MELTING PT° C
		
Phenoxy acetic acid	POA	100
α–Naphthoxy acetic acid	α–NOA	192
β–Naphthoxy acetic acid	β–NOA	155
4–Chlorophenoxy acetic acid	4CPOA	158
2–Methyl–4–chlorophenoxy acetic acid	2M4CPOA (MCPA)	119
24–Dichlorophenoxy acetic acid	24DCPOA (24D)	141
α(24–Dichlorophenoxy) propionic acid	α(24DCPO) P	117
α(24–Dichlorophenoxy)*n*–butyric acid	α(24DCPO) *n* Bu	88
α(24–Dichlorophenoxy)*iso*–butyric acid	α(24DCPO)*iso* Bu	46
2–Methyl–6–chlorophenoxy acetic acid	2M6CPOA	109
26–Dichlorophenoxy acetic acid	26DCPOA	135
w(2–methyl–6–chlorophenoxy)*n*–butyric acid	w(2M6CPO)*n* Bu	Decomp[a]–
245–Trichlorophenoxy acetic acid	245TCPOA	154
245–Trimethylphenoxy acetic acid	245TMPOA	132
246–Trichlorophenoxy acetic acid	246TCPOA	183
35–Dimethyl–4–chlorophenoxy acetic acid	35DM4CPOA	146
α(245–Trichlorophenoxy)*iso*–butyric acid	α(245TCPO)*iso* Bu	92
24–Difluorophenoxy acetic acid	24DFPOA	125
24–Dichlorophenoxy acetic acid	24DCPOA (24D)	141
24–Dibromophenoxy acetic acid	24DBrPOA	151
24–Diiodophenoxy acetic acid	24DIPOA	165

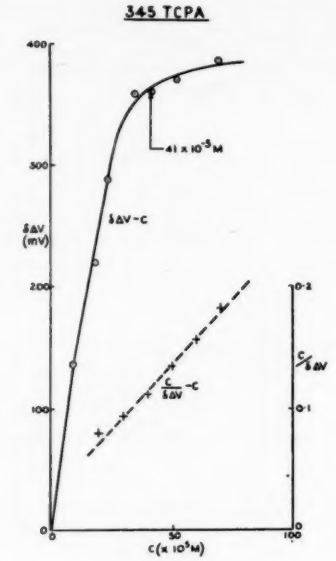
when displaced by a small stream of air. This implies that the surface molecules cohere strongly but as a result of the flexible properties of proteins, a degree of surface mobility is permitted. The original surface properties however are restored when the displacing force is removed. This will be referred to later under "Nature of Films" in the Discussion.

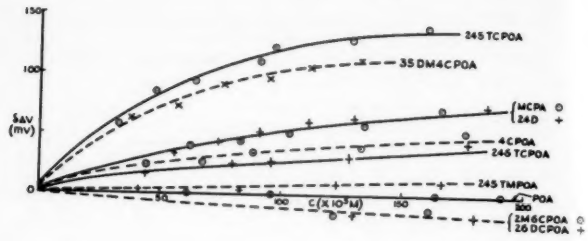
The acids were injected under the monolayers at varying concentrations and Langmuir isotherms were obtained by plotting surface potential change ($\delta\Delta V$) against the concentration of injected acid. In nearly all cases a true Langmuir isotherm was obtained as shown by the production of a straight line on plotting $C/\delta\Delta V$ against C. This is shown in figure 1 for the injection of 345-trichlorophenyl acetic acid beneath oat monolayers at pH 4.30.

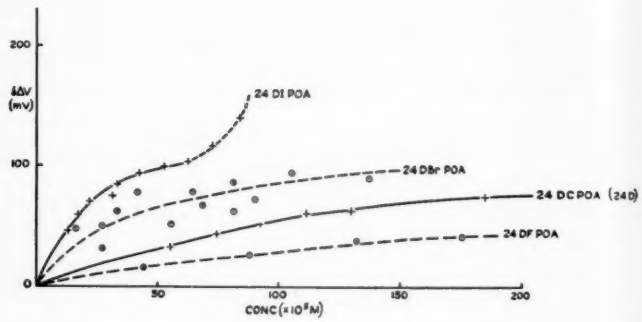
TABLE III

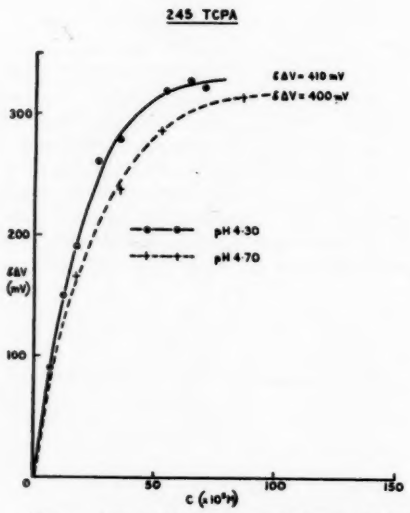
MELTING POINTS AND IDENTIFICATION CODE OF BENZOIC ACIDS

COMPOUND	ABBREVIATION	MELTING PT° C
		
2–Chlorobenzoic acid	2CB	141
24–Dichlorobenzoic acid	24DCB	162
34–Dichlorobenzoic acid	34DCB	204
26–Dichlorobenzoic acid	26DCB	
236–Trichlorobenzoic acid	236TCB	125
245–Trichlorobenzoic acid	245TCB	164









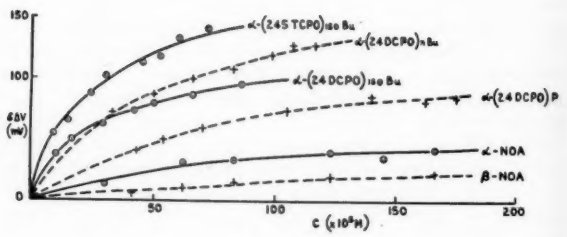


FIG. 1 (*top left*). Oat monolayers injected with 345 TCPA at pH 4.30. 20° C.

FIG. 2 (*bottom left*). Adsorption isotherms for 245 TCPA injected beneath oat monolayers at pH 4.30 and 4.70. 20° C.

FIG. 3 (*top right*). Adsorption isotherms for chloro a nd methylchloro phenoxy acetic acids at pH 4.30. 20° C.

FIG. 4 (*middle right*). Adsorption isotherms for difluoro, chloro, bromo, and iodo phenoxy acetic acids at pH 4.30. 20° C.

FIG. 5 (*bottom right*). Adsorption isotherms for α-and β-naphthoxy acetic acids and chloro phenoxypropionic and butyric acids at pH 4.30. 20° C.

The influence of pH between 4.3-4.7 was always small, figure 2 illustrating the difference in $\delta\Delta V$ for 245-trichlorophenyl acetic acid. With the pKa for all these acids around three, it is assumed that the isotherms result from adsorption of the ion. Isotherms representing the adsorption of each acid at pH 4.30 are drawn in figures 3 to 9 and in each case the surface potential change was assessed at a film area corresponding to $l = 12.0$ cm. This value was plotted against the concentration of acid in the substrate.

Considerable differences exist both in the surface potential changes and the extent of adsorption of the various acids. This is seen in figures 3 to 9 and also in tables 4, 5, and 6 where values of $\delta\Delta V_{41}$, $\delta\Delta V_s$ and the extent of adsorption $\frac{(\delta\Delta V_{41})}{(\delta\Delta V_s)}$ are summarised.

In figure 10 (245-trichlorophenyl acetic acid) it is also seen that the injected acid not only reduces the surface potential of the film but also penetrates into the film, thereby considerably increasing its area. All acids behave similarily but to varying extents; the extent is recorded in tables 4, 5, and 6 (col 5).

These values were obtained by extrapolating the (F-*l*) curves of the monolayers alone and injected with acid to zero compression and by multiplying the value obtained by the width of the trough. The area is thus recorded in square centimeters. The penetration areas were obtained at a fixed degree of adsorption (40 %) and thus represent the total area occupied by a fixed number of adsorbed molecules.

Discussion

Nature of Films. Oat monolayers were selected as reference films since oats[3] were easy to grow throughout the year, their monolayers were reproducible throughout and in Part II these monolayers were shown to adsorb MCPA strongly. Reactions with several acids by less adsorbing monolayers such as were produced from most dicotyledons were difficult to detect.

The nature of the films is unknown. Steady surface potential values however, on films throughout the year indicated that the films were reproducible and this was confirmed by the good agreement obtained between replicate adsorption isotherms using MCPA derived at regular intervals throughout the work.

It is assumed that the films consisted mainly of proteins and lipoproteins from their elastic properties and the observations that their adsorptive capacity fell with increasing pH (2). It is probable therefore that the organic anions (at pH 4.3) react with positive amine and quaternary ammonium salt groups in the film, the reduced adsorption at increasing pH values being due to repulsion of the anions by increasing numbers of carboxyl (COO^-) ions in the film (2).

[3] Var. Victory

Nature of Interactions. Molecular groups involved in the association between a monolayer and adsorbed molecules have been adequately shown by many workers to be hydrocarbon groups (CH_3, phenyl, etc), polar groups (OCH_3, C = O) and ionic groups such as COO^-, $\overset{+}{N}H_3$ and $\overset{+}{N}(CH_3)_3$ (4, 6, 7). The associating forces are proportional to the distance "r" between the groups as follows:

$\frac{1}{r^7}$ for hydrocarbon groups by van der Waals forces

$\frac{1}{r^4}$ for dipole-dipole interactions

$\frac{1}{r^2}$ for ion-ion interactions and for ion-dipole,

$\frac{1}{r^3}$ (6).

It is seen therefore that spacing and stereochemical considerations may be of great importance to the forces of association between two species in particular to the short-range forces $\frac{1}{r^7}$. Accordingly a change in the ionization, dipolar charge, or number of hydrocarbon groups greatly influences the extent of formation or stability of a complex in these laboratory systems or natural systems such as membranes and enzymes.

It is important to note that although there is a large degree of cohesion between the surface molecules, there is also some molecular flexibility shown by the recoil of talc as already described. Thus flexibility may well facilitate the formation of complexes between surface and injected organic acids.

The adsorption of these organic acids therefore may reasonably be assumed to occur through ion-ion interactions reinforced by van der Waals forces involving hydrocarbon groups. The speed and reversibility of adsorption supports this view and in plant growth regulation causes the author to favor the physicochemical association of chemicals with natural substrates as opposed to reactions involving covalent bonds.

Thus the interactions studied here may also be regarded as an indication of the extent to which partition of organic acids between aqueous substrate and surface film might occur.

Complex formation between film and injected acid manifests itself in two ways:

A, Surface potential changes $\delta\Delta V$.

B, Expansion of the monolayer. By combining these two types of result, indications can be obtained

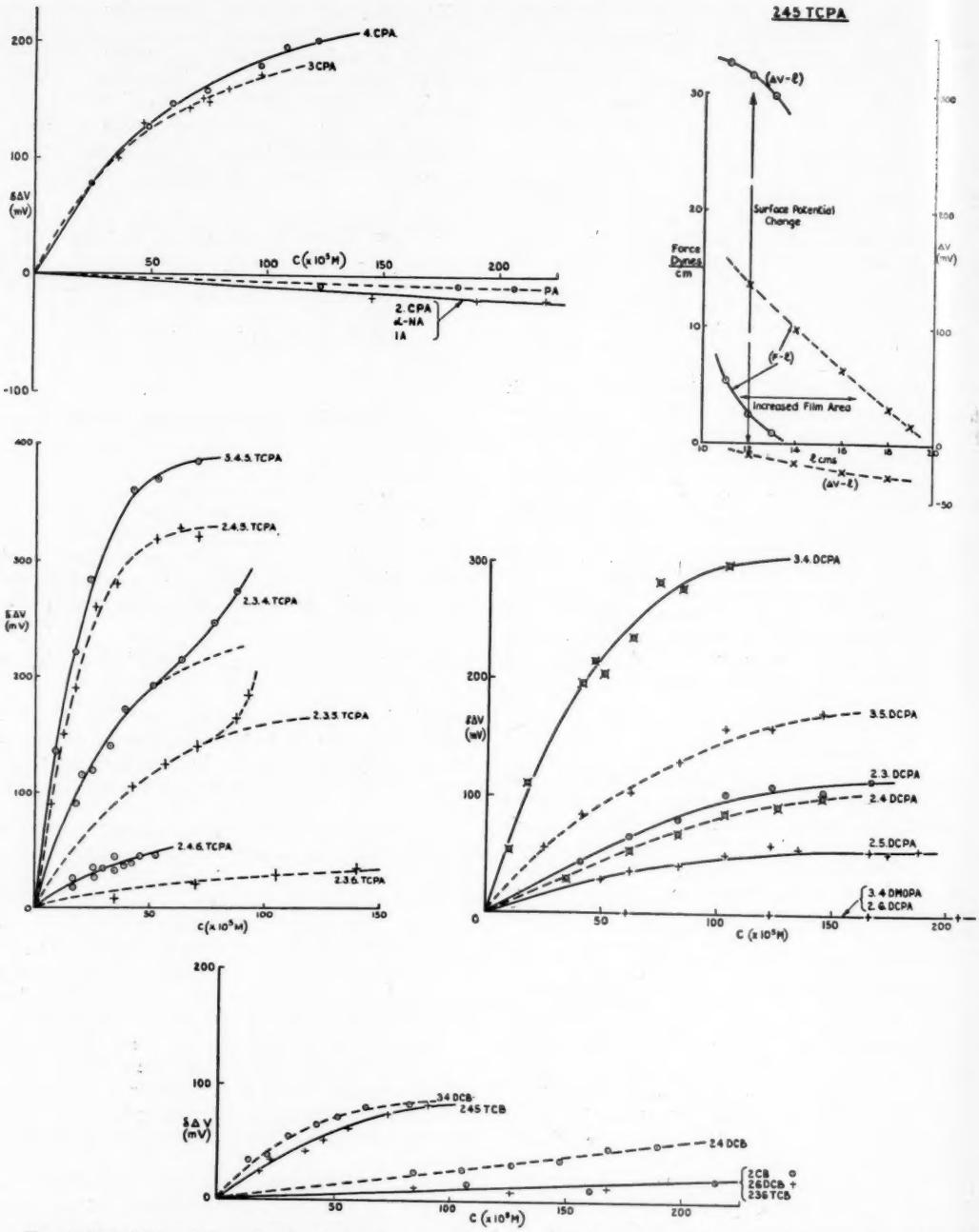


FIG. 6 (*top left*). Adsorption isotherms for monochlorphenyl acetic acids, phenyl, α-napthalene and indole acetic acids at pH 4.30. 20° C.

FIG. 7 (*middle right*). Adsorption isotherms for dichlorophenyl acetic acids at pH 4.30. 20° C.

FIG. 8 (*middle left*). Adsorption isotherms for trichlorophenyl acetic acids at pH 4.30. 20° C.

FIG. 9 (*bottom*). Adsorption isotherms for chlorobenzoic acids at pH 4.30. 20° C.

FIG. 10. (*top right*). Changes in surface area and potential due to injection of 60×10^{-5} M 245 TCPA beneath oat monolayers at pH 4.30. 20° C.

of the mode of adsorption. The amount of adsorption was derived only from the surface potential determinations.

A. Mode of Adsorption. It is not possible to calculate the area occupied by the adsorbed molecules in terms of square Angstroms per molecule but the penetration area for the acids can be compared if the comparison is made at a fixed degree of adsorption. Thus the (F-*l*) curve obtained by injecting an acid concentration to give 40 % adsorption was extrapolated to zero compression and the penetration area was obtained by subtracting from this area the extrapolated value of the monolayer alone (see fig 10). Using this method however, it has not been possible to report values of acids for which 40 % coverage was not obtained.

Although the comparison is made at 40 % adsorption, only a proportion of the adsorbed molecules may have penetrated the monolayer, the rest remaining in the water substrate. It is believed that the difference in penetration areas reflects the ability of the acids to penetrate the surface films rather than the actual adsorbed area of completely penetrated molecules. Thus minor area differences only are found between 24-dichloro and 24-diiodophenoxyacetic acids (table 5, col 5) in spite of the much greater size of the iodine atoms.

Phenoxy Compounds. Comparison of tables 4 and 5 indicates that phenoxy compounds in general gave penetration areas much less than corresponding phenyl acetic acids. Even α- and β-naphthoxy acetic acids in which there are bulky fused ring systems expanded the films insignificantly. A reason for this is given by measurements of $\delta\Delta V_s$. Recalling that these values are largely determined by the vertical disposition of the chlorine atoms relative to the carboxyl group, it was found that $\delta\Delta V_s$ for the phenoxy acids was less than that of the phenyl acetic acids. The phenolic oxygen with affinity for the water surface presumably influenced their mode of penetration. A two point contact with the surface is envisaged viz., the $-COO^-$ and the phenolic oxygen are in the water phase (figure 11d). This model is confirmed by the close agreement in $\delta\Delta V_s$ values for 245-trichlorophenoxy and benzoic acids suggesting that the distance between the ring chlorine atoms and the COO^- group in the two acids when adsorbed, is similar (fig 11 d–e). This is further confirmed by comparing $\delta\Delta V_s$ for 4-chlorophenoxy acetic acids (120 mV) with that of 4-chlorophenyl acetic (350 mV), since the two point contact would lessen the vertical separation of the chlorine and carboxyl groups. The influence of the oxygen atom on penetration in phenoxy compounds may also explain the differences found between methyl and chloro derivatives and between the acetic acids and the propionic and butyric acids. Thus methyl compounds penetrated more readily than corresponding chloro compounds as seen in MCPA and 24D or (assuming their adsorptions are similar) between 245-trimethyl and 245-trichlorophenoxy acetic acids (table 5, col 5). Although not strictly comparable, the pene-

Table IV

Adsorption Characteristics of Indole, α-Naphthyl and Phenylacetic Acids pH 4.30

Compound	$\delta\Delta V_{41}$	$\delta\Delta V_s$	Extent of adsorption (%) $\delta\Delta V_{41}/\delta\Delta V_s$	Penetration area at 40 % adsorption
PA	−2			
IA	4			
α–NA	−2			
2CPA	−6			
3CPA	114	275	42	45
4CPA	114	350	33	42
23DCPA	44	170*	26*	38
24DCPA	35	170*	21*	48
25DCPA	23	90	26	17
26DCPA	−8			
34DCPA	195	500	40	55
35DCPA	81	300	27	45
234TCPA	171	400	43	42
235TCPA	102	330	31	56
245TCPA	300	410	73	53
236TCPA	14			
246TCPA	41	110**	37	
345TCPA	362	480	75	50
34DMOPA	−9			

* Based on estimated value.
** Limited number of points due to insolubility, hence less reliable.

TABLE V

ADSORPTION CHARACTERISTICS OF α-AND β-NAPHTHOXY AND PHENOXYACETIC ACIDS pH 4.30

COMPOUND	$\delta\Delta V_{41}$	$\delta\Delta V_s$	EXTENT OF ADSORPTION (%) $\delta\Delta V_{41}/\delta\Delta V_s$	PENETRATION AREA AT 40% ADSORPTION
POA	2			
α–NOA	24			
β–NOA	8			
4CPOA	18	120	15	16.2
2M4CPOA (MCPA)	27	130	21	30.0
24DCPOA (24D)	27	140	19	21.0
α(24DCPO)P	39	125	31	22.0
α(24DCPO)*n* Bu	81	180	45	39.0
α–(24DCPO)*iso* Bu	75	120	63	46
2M6CPOA	−3			
26DCPOA	−3			
w(2M6CPO)*n* Bu	20	Erratic results		
245TCPOA	72	220	33	22
245TMPOA	3			
246TCPOA	18	45*	40	22
35DM4CPOA	64	200	32	43
α(245TCPO)*iso* Bu	112	160	70	35
24DFPOA	15	88	17	10
24DCPOA (24D)	27	140	19	21
24DBrPOA	60	120	50	18
24DIPOA	92	152	60	28

* Limited range of points due to insolubility hence less reliable.

tration area of 35-dimethyl-4-chlorophenoxy acetic acid was twice that of 245-trichlorophenoxy acetic acid. It is reasonable to assume that methyl groups leave the water surface more easily than chlorine atoms.

Similarly, table 5 indicates that lengthening of the acetic acid chain by a $-CH_2$ group in the propionic acid had no effect on penetration or $\delta\Delta V_s$ whereas the *n*-butyric acid showed increase of penetration and of $\delta\Delta V_s$. This suggests that the longer hydrocarbon chain is able to overcome the affinity between the water and the phenolic oxygen.

METHOXY GROUPS. Methoxy groups in 34-dimethoxyphenol acetic acid prevented penetration of the molecule and in spite of their high electronegativity, produced little or no surface potential change. This is possibly due to a radical change in the mode of adsorption as a result of the affinity for water of the methoxy groups (see fig 11c).

CHLORINE SUBSTITUTION. As there are no chlorine atoms in phenyl and phenoxy acetic acids, little or no surface potential changes were found even at high concentrations. Introduction of electro-negative groups into the aromatic ring, however, markedly influenced the vertical component of the dipole and the $\delta\Delta V_s$ values noted depend on the disposition and number of these groups. Hence $\delta\Delta V_s$ was almost zero for 2-chlorophenyl acetic acid where the chloro group is close to the carboxyl ion but large values (275, 350 mV, resp.) were found for 3-and 4-chlorophenyl acetic acid. (fig 6).

Figures 7 and 8 also show isotherms for phenyl acetic acids in which both 3 and 4 positions of the ring are occupied. Very large $\delta\Delta V_s$ values resulted even when the 2 position is also filled. This is no doubt due to the production of a strongly negative dipole at maximum separation from the negatively charged carboxyl ion. This is plainly seen in fig 7 where isotherms for the dichlorophenyl acetic acids are presented, the 3,4 compound being unique, and in figure 8 where $\delta\Delta V_s$ for 245-trichlorophenyl acetic acid (410 mV) is greater than that of the 235 (330 mV).

Substitution of chlorine in the 2 position to the side chain however resulted in some reduction in $\delta\Delta V_s$ e.g. 34-dichloro (500 mV) and 234-trichloro (400 mV) and also 3-chloro (275 mV) and 23-dichloro (170 mV). But 26 substitution produced a much greater reduction. Thus compare the following trichloro-substituted phenyl acetic acids -235- (330 mV) with 236-(almost zero) and 245-(410 mV) with 246-(110 mV).

In the disubstituted acids, no measurable surface

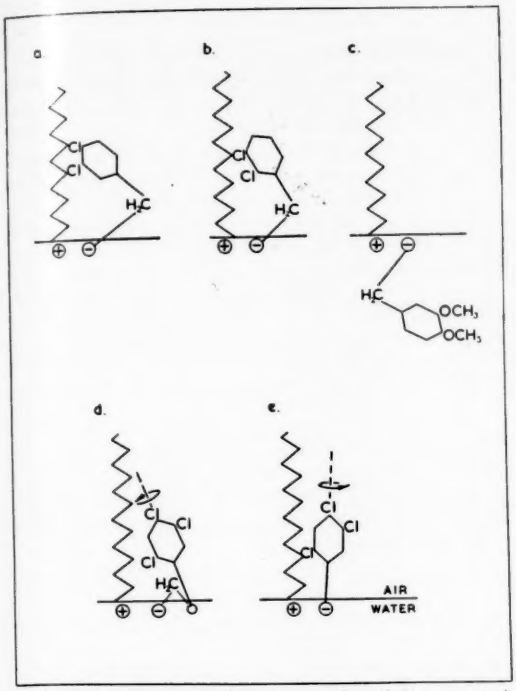


FIG. 11. Diagrammatic representation of the suggested mode of adsorption of phenoxy and phenyl acetic acids and benzoic acids. a. 34DCPA. b. 23DCPA. c. 34DMOPA. d. 245TCPOA. e. 245TCB.

potential change was found when the 2 and 6 positions were filled with chlorine and methyl groups in either the phenyl, phenoxy, or benzoic acid series. Moreover, even at high concentrations of injected acid, no significant penetration was obtained. This is surprising in view of the apparently larger size of these disubstituted acids compared with the monosubstituted compounds. Low penetration was also obtained with the 236-and 246-trisubstituted acids.

The following illustrations which are projections of three dimensional models are thought to be representative of the systems under discussion.

B. AMOUNT OF ADSORPTION. The amount of adsorption is calculated from a curve in which the change of surface potential is plotted against the concentration of injected acid. It cannot be derived directly from $\delta\Delta V$ values however, since as seen in the last section, these are a function of the structure of the injected molecule as well as the degree of adsorption. For this reason $\delta\Delta V$ values at 41×10^{-5} M are made relative to $\delta\Delta V_s$ values (surface saturation) thus eliminating the structural component.

As seen in the preceding section, a number of acids produced little or no surface potential change. These include the naphthalene and naphthoxy acetic acids, the phenoxy and phenyl acetic acids, and 2 chloro and 26 dichloro substituted acids. It is therefore not possible to determine their degree of adsorption by this method and these acids will be made the subject of a later paper.

GENERAL. Comparison of tables IV and V indicates that phenyl acetic acids are more adsorbed than phenoxy acetic acids. The difference is particularly marked with the 4-chloro and 245-trichloro derivatives. It is also interesting that the adsorption of the 245-trichloro-substituted acids falls from 73 % for the phenyl to 33 % for the phenoxy and 27 % in the benzoic acids. The excessive falls in adsorption for these tri-substituted acids may indicate that the phenoxy and benzoic acids are less flexible than the phenyl acids in the adsorbed state (see fig 11a, b, d, e) and hence the adsorption complex does not easily accommodate the third chlorine in the 2 position. The adsorption of 234,- 235-, and 245-trichlorophenyl acetic acids were all high.

2-AND-26-SUBSTITUTION. 2-Substitution reduced adsorption even in the phenyl acetic acids where it is seen from table IV that the 23-, 24-, and 25-dichloro acids were all less adsorbed than the 3-and 4-monosubstituted derivatives. The effect of 2-substitution therefore is not fully understood. In all three series 26-substitution apparently reduced adsorption to zero. This also applied to 236-tri-substitution but there was a measure of adsorption in the 246-trichloro acids. (The values for the latter must not be taken literally as it was not possible to construct adsorption isotherms for the usual range of concentrations owing

TABLE VI

ADSORPTION CHARACTERISTICS OF BENZOIC ACIDS pH 4.30

COMPOUND	$\delta\Delta V_{41}$	$\delta\Delta V_s$	EXTENT OF ADSORPTION (%) $\delta\Delta V_{41}/\delta\Delta V_s$	PENETRATION AREA AT 40 % ADSORPTION
2CB	4			
24DCB	11			
34DCB	64	135	47	25
26DCB	4			
236TCB	4			
245TCB	52	194	27	18

to their limited solubility in acetate buffers). Similarly, there was no apparent adsorption with 2-chlorophenyl or 2-chlorobenzoic acids. However, the absence of surface potential changes is not conclusive evidence for low adsorption and the low values may equally result from the disposition of the chlorine atoms in these compounds.

PHENOXY COMPOUNDS. In the phenoxy acetic acid series, it is interesting that the adsorption characteristics of 24D and MCPA were identical. The adsorption values of 19 and 21, respectively, were intermediate in magnitude and this may be a clue to their high biological activity. While high adsorption may lead to excessive loss at physiologically inactive sites, low adsorption may prevent the growth regulating reaction at the enzyme site. In this respect it may be significant that the highly active growth regulators 4-chloro, 24-dichloro, 2-methyl-4-chloro, 245-trichlorophenoxy acetic acids, and α(24-dichlorophenoxy) propionic acid all had intermediate adsorption values of 15 to 33. It is interesting that the order of adsorption of some phenoxy acetic and benzoic acids to oat monolayers agrees qualitatively with that found for the same series by Leopold (5) using steam-killed bean leaves. Lengthening the acetic acid chain to give propionic and butyric acids increased the adsorption value as expected. It was surprising however to note the large increase in adsorption for the α-phenoxy isobutyric acids, 63 % and 70 % adsorption being obtained for the 24-dichloro and 245-trichloro derivatives, respectively. It is interesting to speculate that this high adsorption is a contributing factor in their biological inactivity.

While a progressive increase in adsorption for 24-difluoro to 24-diiodophenoxy acetic acids was anticipated it is surprising to find such a marked increase when chlorine is replaced by bromine. The behavior of the dibromo compound however was anomalous throughout. Thus its adsorption greatly exceeded that of the dichloro compound and yet its penetration area and $\delta\Delta V_s$ value was less.

It is not possible to complete the discussion of this work until the adsorption properties of the unsubstituted and 26-disubstituted compounds have been studied and until biological data are available on the phenyl acetic acids. It is proposed to present these in the final Part IV of this series.

SUMMARY

In Part II of this series, a broad correlation was found between the resistance of plants to 2-methyl-4-chlorophenoxy acetic acid (MCPA) and their ability to adsorb the growth regulator.

The possibility is investigated in this paper of the biological activity of compounds on a given plant species also being limited by their adsorption to sites not concerned in the physiological response. The measurement of adsorption values of a series of chloro- and methyl-phenoxy and phenyl acetic acids and benzoic acids to monolayers from oat leaves is reported and the results are shown to indicate how the acids are adsorbed and in what relative quantities.

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ON THE THEORY OF OSMOTIC WATER MOVEMENT[1]

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In explaining osmotic movement of water, it is customary in plant physiology to regard water movement between cells as taking place along gradients of diffusion pressure deficit or suction force. Efforts to describe water movement quantitatively are based on the assumption that the rate of water movement between two cells should be proportional to the difference between the diffusion pressure deficits (or suction forces) of the two cells. The purpose of this paper is to consider some aspects of the theory of osmotic water movement which arise out of this elementary rate problem, and indicate some fundamental difficulties in the concepts currently in use. First we shall examine critically the meaning of the concepts "suction force" and "diffusion pressure deficit". Then their applicability as parameters of osmotic water movement will be considered. Finally, the value of rate measurements in indicating the mechanism of osmosis will be reviewed, and a hypothesis concerning the mechanism of osmotic flow will be presented.

SUCTION FORCE. The suction force of an osmotic unit such as a cell, is defined (1, 54) in two distinct ways. First, it is taken to be equal to the osmotic potential of that solution which, at atmospheric pressure, would be in osmotic equilibrium with the unit in its existing state. By osmotic potential (Π, atm)[2] we mean the pressure which would have to be applied to a solution to maintain osmotic equilibrium between it and pure water, through an ideal semipermeable membrane. The term "osmotic potential" is used for this potential pressure, commonly called "osmotic pressure", to minimize confusion between it and actual existing hydrostatic pressure, designated P (atm).

Second, the suction force (S) of an osmotic unit is stated (1, 54) to have the value

$$S = \Pi - P \qquad I$$

Π and P refer to osmotic potential and pressure within the osmotic unit. This equation usually is not accurately justified, so that the student is obliged to accept on faith that S defined by equation I has the same value as when defined by the equilibrium criterion first mentioned. The meaning of equation I can be seen by comparing its transposed form

$$P_1 = \Pi_1 - S_1 \qquad II$$

with the demonstrable fact that the pressure required to maintain osmotic equilibrium between two solutions must be equal to the difference in their osmotic potentials,

$$P_1 = \Pi_1 - \Pi_2 \qquad III$$

(This relationship is readily derived from the basic equation for osmotic equilibrium, equation IV, to be discussed below.) It is thus seen that S_1 must have the same value as Π_2, the external solution which would be in equilibrium with osmotic unit 1 with its osmotic potential Π_1 and pressure P_1. Equation I is, therefore, consistent with the experimental definition of S first mentioned; it defines a state of the osmotic unit useful in predicting whether or not equilibrium can obtain between it and any second unit. From equation II it is readily shown, in a manner similar to that used above for an external solution, that any two osmotic units can be in equilibrium if and only if they have the same value of S.

Diffusion pressure deficit is widely accepted as having the same meaning and quantitative value as suction force. A different concept is involved, however, which must now be examined.

DIFFUSION PRESSURE. Osmosis is commonly held by plant physiologists to be a diffusion process, which may explain why the terms to be discussed are so popular. Diffusion pressure deficit is defined as follows:

"Diffusion pressure deficit of water . . . . is the amount by which its diffusion pressure is less than that of pure water at the same temperature and under atmospheric pressure" (34).

Diffusion pressure is a hypothetical quantity which is supposed to govern the rate of diffusion out of a solution. The quantitative basis of diffusion pressure, according to modern usage throughout plant physiology, is the same parameters as are used to define S. This is shown, for example, by the statement, "The osmotic pressure is an index indicating quantitatively the amount by which diffusion pressure of water in the solution is less than that of pure water at the

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[2] An appendix defining all mathematical symbols used here, appears at the end of the paper.

same temperature and under atmospheric pressure" (34).

A consequence of basic considerations in thermodynamics, which may be found in any modern treatise of the subject, is that the pressure (P_1) required to maintain ideal osmotic equilibrium between two solutions is given by

$$P_1 = \frac{RT}{\bar{V}} \ln \frac{p_2}{p_1} \qquad \text{IV}$$

where p_1 and p_2 are the partial vapor pressures[3] of the solvent in solutions 1 and 2, and the positive pressure P_1 is applied to solution 1; $\bar{V}$ is the partial molal volume of the solvent. When solution 2 is pure solvent, then P_1 is equal to the osmotic potential (Π_1) of solution 1, as is well known. Since the partial vapor pressure of solvent approaches zero as its concentration tends to zero, it follows that the osmotic potential of a solution increases without limit as the solvent concentration approaches zero as a limit. That this must be so, can be seen by considering, for example, a sample of pure glycerol separated from water by a membrane permeable only to water. To maintain equilibrium, it would be necessary to increase the diffusion rate of water out of the glycerol until it equalled the rate of diffusion of water into the glycerol; but pure glycerol would contain no water at all, so it would be impossible to maintain equilibrium by any pressure.

The diffusion pressure of pure water must be the difference between the diffusion pressures of water and of a solution containing no water at all, in other words it must be equal to the diffusion pressure deficit of a solution containing no water, or to its osmotic potential if it is at atmospheric pressure. It follows from what has been said above that the diffusion pressure of pure water must be infinitely large: this has been remarked by Crafts, Currier, and Stocking (12). The diffusion pressure deficit of a solution (the difference between diffusion pressures of pure water and of water in the solution) is hence the difference between an infinitely large quantity and some other quantity, and therefore can have no numerical value. It is not possible to obviate this difficulty in the fashion, which seems to have been accepted almost throughout plant physiology, of setting the diffusion pressure of pure water equal to zero (12), as this amounts to setting infinity equal to a finite number. Diffusion pressure deficit is self-contradictory because the definition of this quantity in terms of diffusion pressures, and of osmotic potential and hydrostatic pressure (equation I), is not consistent. In other words, there cannot be a diffusion pressure which can be defined by the statements quoted above, and if there is no such quantity as diffusion pressure, there cannot be a diffusion pressure deficit. The concept is simply not valid.

The idea of diffusion pressure as explaining osmosis was originated by Haldane (20), whose theoretical work is cited as the basis for introducing the concept of diffusion pressure into plant physiology (33). It is important to note that in Haldane's theory of osmosis, the diffusion pressure difference between a solution and pure solvent was explicitly *not* equal to the osmotic potential of the solution, and so was not identical to the diffusion pressure deficit as defined by more recent authors (equation I). The concepts of diffusion pressure are, consequently, also not the same. Thus in modern usage diffusion pressure deficit is not only self-contradictory, but it conflicts with the justification for assuming that a diffusion pressure governs osmosis.

Haldane's theory of diffusion pressure and osmosis is inconsistent with equation IV, and therefore is thermodynamically unsound. Hence it would be fruitless to attempt to rescue the concept of diffusion pressure deficit by giving up equation I and substituting Haldane's definition. Neither his nor the modern concept of diffusion pressure appears to have any physical meaning, and neither concept is any longer in use outside of plant physiology. It seems inevitable that the use of diffusion pressure and diffusion pressure deficit in plant physiology will have to be abandoned.

Suction force does not contain the contradictions just discussed, as it involves no concept analogous to diffusion pressure, but is simply tied consistently to equation I and to the state of osmotic equilibrium between a cell and a solution, as indicated above. The term (not the concept) has been justly criticized (34). In the remainder of this paper we shall employ the generalized term "water deficit" (S), defined by equation I, for this equilibrium parameter. Since Π and P in equation I are linearly connected with differences in the chemical potential of the solvent, S is linearly related to thermodynamic description of an osmotic system, as presented, for example, by Broyer (5). S can be used just as exactly as chemical potential in dealing with osmotic equilibrium through semipermeable membranes, as long as active transport of water is not involved.

WATER MOVEMENT RATES. Since water deficit (S) correctly defines states of osmotic equilibrium, it should predict correctly the direction of water movement in systems not at equilibrium. The assumption that the *rate* of water movement between two osmotic units is proportional to the water deficit *difference*,

$$v_{1\to 2} = K_0 A(S_2 - S_1) = K_0 A[(\Pi_2 - \Pi_1) - (P_2 - P_1)] \qquad \text{V}$$

has little more than an intuitive basis in the plant physiological literature, even though it was formulated as early as 1921 by Ursprung and Blum (55). There is some evidence that water movement in or into plant tissues may follow equation V (3, 4, 6, 47), although not all of the results are in close agreement with it,

[3] Or fugacities, for solvents whose vapor does not behave as an ideal gas. For the original derivation, see (15).

and none cover a wide range of rates. Equation V has been supported by making an analogy between osmotic potential and electrical potential, to which current is proportional (3, 19, 23, 24). Water movement is then thought of as determined by a potential drop divided by a resistance to movement. This led to the attractive idea that in steady water flow through the plant, when total flow in each tissue of the conducting system must be the same, the potential drop in each tissue must be proportional to the resistance of that tissue,

$$\nu = \frac{S_2 - S_1}{r_{21}} = \frac{S_3 - S_2}{r_{32}} = \frac{S_4 - S_3}{r_{43}} \qquad \text{VI}$$

which was suggested by Huber (24) and Gradmann (19) and was formulated by van den Honert (23). However, rates are not proportional to differences in potential with most other processes than electric current, for example, reaction rates and chemical potentials. The analogy provides no satisfying or reliable basis for belief in the principle embodied in equations V and VI.

In the literature of zoophysiology, equation V is known as "Starling's hypothesis" for movement of water between capillaries and tissue fluids. (40). A certain amount of quantitative support for it has been obtained in recent years. It has been derived theoretically using thermodynamics of irreversible processes (50), but this derivation rests on an assumption about rate proportionality which may have only limited application (13). Equation V has been derived for a diffusion process upon thermodynamic (7) and kinetic (29) grounds; in these cases it arises as an approximation.

That equation V cannot be true in general, for rates of osmotic water movement can be seen by the following example. Consider, as shown in figure 1, an osmotic system composed of pure glycerol separated from pure water by an air space, which acts as an osmotic membrane because water vaporizes into it but glycerol does not. The osmotic potential, and therefore water deficit, of pure glycerol is, as brought out above, infinitely large, but it is evident that the rate of transfer of water from the pure water into the glycerol will have a finite value; it will be equal to the rate at which water evaporates from the water surface and diffuses through the air into the glycerol. This rate will be dependent upon temperature and the cross section and length of the air space, and is determined by diffusion parameters which are not proportional to the (infinite) water deficit difference.

If osmotic movement of water through a membrane is regarded as a diffusion process, as it commonly is in plant physiology, then the above considerations will apply also to glycerol and water separated by a semipermeable membrane; the rate of diffusion of water through the membrane cannot be proportional to the water deficit difference, which is infinite. Before examining the relationship which would be expected between diffusion and rate of osmotic water movement, we shall first consider one aspect of water movement in plants for which equation VI is clearly unacceptable.

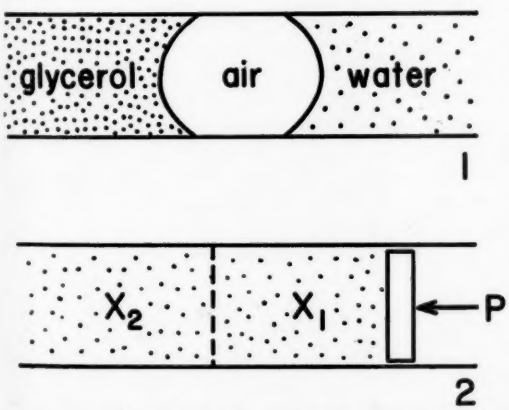

FIG. 1 (*above*) and FIG. 2 (*below*). Model osmotic systems, described in the text.

TRANSPIRATION. Equation IV is applicable to states of equilibrium between liquid water at pressure P_1 and water vapor at partial pressure p_2, if p_1 is the vapor pressure of liquid water at atmospheric pressure. The equilibrium pressure P_1 is the pressure which would have to be applied to make the vapor pressure of water p_2 at the same temperature. By analogy with equation I, $-P_1$ has been called the diffusion pressure deficit or suction force of water vapor (S′) at partial pressure p_2. In conceiving of water movement in plants as involving successive potential drops, Gradmann (19) was led to assume that the rate of transpiration should be proportional to the suction force difference between leaf cells and aerial water vapor. More recent authors (2, 23, 45) have included transpiration as the last term in equation VI, where S_4 becomes the suction force of the air, and r_{43} the transpiration resistance. It can be seen from equation IV that this means that the rate of water loss by a plant should be logarithmically dependent upon the water vapor pressure difference between the leaves and the atmosphere, whereas in fact it is nearly linearly dependent upon the vapor pressure difference (30). The expectation of proportionality between S′ difference and transpiration rate is a clear example of confusion between rate and equilibrium parameters, which has serious consequences[4]. It would mean, for example, that transpiration rate

[4] This error is related to the cause of failure of the "diffusion pressure deficit" concept. In the vapor phase, diffusion pressure deficit as used by plant physiologists (S′) is clearly *not* equal to the difference in diffusion pressures [partial pressures, see (34)], so the term is obviously erroneous.

would increase without limit into perfectly dry air, whereas in fact it should never be more than about twice the rate found in air of 50 percent relative humidity (other conditions constant).

There is another factor involved in transpiration rates which S′ difference cannot cope with, and which is particularly significant at high relative humidities where S′ and diffusion gradients are nearly proportional. This is that in sunlight, the temperature of the leaf can be higher than that of the surrounding air, increasing the internal water vapor pressure of the leaf and therefore the diffusion gradient outward; this effect is much larger than the small effect (proportional to absolute temperature) on S′ of the leaf, hence it is possible with nearly the same S′ difference to have quite widely different transpiration rates.

Therefore, transpiration cannot correctly be included in equation VI. A kinetically reasonable expression for transpiration rate must be based on diffusion coefficients and water vapor pressures (48) not on S′. Suction force of the air is useful only in evaluating states of equilibrium, that is, of zero transpiration.

Osmosis as a Diffusion Process. Regardless of whether or not a net movement of water is occurring, diffusion takes place continually across a membrane permeable to water, in the sense that water molecules are constantly moving through it in both directions. If osmotic water movement across a membrane is due to diffusion, it must represent the difference between diffusional movement of water molecules in one direction and in the other. A simple way of evaluating the molecular flux in one direction is to imagine diffusion from water or a solution into pure solute, as with the glycerol example considered above. We shall start with evaporation of solvent from a solution, which is proportional to the partial vapor pressure of the solvent and hence, for an ideal solution, proportional to the mole fraction (X) of solvent in the solution, according to Raoult's Law. Diffusion of solvent across a membrane into pure solute is an analogous phenomenon, and for an ideal solution the rate of outward diffusion (d) should be proportional to solvent mole fraction (X) and membrane area (A), and inversely proportional to membrane thickness (l), which constitutes the length of the diffusion path

$$d = \frac{k_d A}{l} X \qquad \text{VII}$$

where k_d is a diffusion coefficient. We wish to know how the rate of outward diffusion will depend upon both X and any hydrostatic pressure (P) which may be applied. One way of evaluating this (there are others, stricter but more lengthy) is with an osmotic system like that shown in figure 2, in which solution 2 of composition X_2, at atmospheric pressure, is separated by a semipermeable membrane from solution 1 of composition X_1, to which a hydrostatic pressure P_1 is applied so as to maintain equilibrium. As is usual in discussions of osmosis, it will be assumed that to maintain equilibrium, P has to be adjusted until the rate of outward diffusion of water molecules from solution 1 is equal to the rate of outward diffusion from solution 2. This latter rate is given by equation VII. The required pressure is given by equation IV, which can be written

$$P_1 = \frac{RT}{\overline{V}} \ln \frac{X_2}{X_1} \qquad \text{VIII}$$

for an ideal solution. Incorporating equation VII for the solution at atmospheric pressure,

$$P_1 = \frac{RT}{\overline{V}} \ln \frac{l \cdot d}{k_d A X_1} \qquad \text{IX}$$

then

$$d_1 = \frac{k_d A}{l} X_1 e^{P_1 \overline{V}/RT} \qquad \text{X}$$

The difference between diffusional fluxes in forward and reverse directions across a membrane separating any solutions 1 and 2 is then

$$\nu_{1\to 2} = d_1 - d_2 = \frac{k_d A}{l} (X_1 e^{P_1 \overline{V}/RT} - X_2 e^{P_2 \overline{V}/RT}) \qquad \text{XI}$$

This is the basic equation for osmotic diffusion of water. A similar equation was derived, by a more detailed argument, by Laidler and Shuler (29). We may modify it, for the relatively low pressures involved in most plant cells, by expanding e^a as the series $(1 + a + a^2/2! + \ldots)$, and neglect terms from a^2 on, because $P\overline{V}/RT$ will be a number considerably less than one. Then

$$\nu_{1\to 2} = \frac{k_d A}{l} (X_1 + \frac{P_1 \overline{V} X_1}{RT} - X_2 - \frac{P_2 \overline{V} X_2}{RT}) \qquad \text{XII}$$

We shall now transform this equation by adding and subtracting one within the brackets, then multiplying and dividing by $\overline{V}/RT$. We get

$$\nu_{1\to 2} = \frac{k_d A \overline{V}}{lRT} \left[\frac{RT}{\overline{V}} (1 - X_2) - \frac{RT}{\overline{V}} (1 - X_1) - (X_2 P_2 - X_1 P_1) \right] \qquad \text{XIII}$$

Here the quantities $(1 - X)/\overline{V}$ are, for dilute solutions, approximately equal to the volume molar solute concentrations c, and the resulting terms cRT ap-

proximate the osmotic potentials Π of the two solutions, according to the well-known van't Hoff relationship. Also, if the solutions are dilute, X_1 and X_2 will be very close to one, so we may take the coefficients of the P terms as equal to one. This leads to

$$v_{1\to 2} = \frac{k_d A \overline{V}}{lRT}[(\Pi_2 - \Pi_1) - (P_2 - P_1)] \quad \text{XIV}$$

It can thus be seen how equation V arises as an approximation, good only for dilute solutions and low pressures, of the more general expression for diffusion of water across membranes (equation XI). Actually, the difference between equation V and XI will be of minor quantitative importance under ordinary conditions, but it is not likely to remain so under conditions of low water content or high moisture stress, such as in seeds or desert plants. Moreover, it is important to recognize that in principle, the parameters of osmotic equilibrium (S) and osmosis rate are divergent.

Comparison of Diffusion and Osmosis. By comparing equation XIV with equation V it can be seen that if osmosis is, as assumed, a diffusion process, then for any given membrane at a particular temperature, the osmotic permeability (K_o) should be related to the water diffusion permeability (K_d) by the factor $\overline{V}/RT$, which has a value of about 7.5×10^{-4} atm^{-1} in the physiological range of temperature.

Diffusion permeability to water has been measured for several plant cells or tissues using isotopically labelled water (DHO or THO). In most of the cases the simplest comparison of diffusion with osmotic permeability can be made in terms of the half-times for diffusion exchange ($t_{½\,d}$) and osmotic exchange ($t_{½\,o}$) of water when the cell or tissue is transferred from one situation to another. Philip (42, 43) showed that for a cell or tissue of any particular geometry, and under certain assumptions, these half-times are related to the respective permeability coefficients as follows:

$$\frac{t_{½\,d}}{t_{½\,o}} = \frac{k_o(\epsilon + \Pi_o)}{k_d} \quad \text{XV}$$

Here ϵ is the elastic modulus of the cell wall (atm) and Π_o the osmotic potential at zero turgor pressure[5]. Accordingly, we shall compute the ratio of permeability coefficients from the formula

$k_o/k_d = t_{½\,d}/t_{½\,o}\,(\epsilon + \Pi_o)$.

Five available cases will be examined.

A. Ordin and Bonner (36) found $t_{½}$ values of 8 to 9 minutes for diffusion of deuterium-labelled water into and out of Avena coleoptile sections 5 mm long. They also determined $t_{½}$ for osmotic equilibrium, by following the change in length when a section was transferred into or out of 0.4 M mannitol, and found a value of about eight minutes. From their data (35) $(\epsilon + \Pi_o)$ can be estimated as 150 atm. From these figures $K_o/K_d = 7 \times 10^{-3}$ atm^{-1}.

B. Wartiovaara (61), who was the first to use labelled water to study water permeability of a plant cell, determined the course of outward diffusion of DHO from individual cells of the alga *Tolypellopsis stelligera* (= *Nitellopsis obtusa*). He found half-times of approximately 0.7 minutes, corrected for build-up of external DHO concentration. The osmotic permeability of similar cells of Tolypellopsis was determined by Palva (39), using a density-gradient method, with half-times of about 2.0 minutes. From his data ϵ is found to have a value of about 75 atm; Π_o is not given but can be taken as about eight atm from data of Collander (9). Then $K_o/K_s = 4 \times 10^{-3}$ atm^{-1}.

C. Collander (11) determined the permeability of single cells of *Nitella mucronata* to DHO, and reported a permeability coefficient (K_d) of 6.33×10^{-4} cm sec^{-1}. He also (10) found half-times of 5 to 8 seconds for osmotic exchange of water when cells of this alga were transferred between water and 0.2 M solutions of sucrose or urea. Π_o for the cells is given as about seven atm (10), but it is not possible to deduce ϵ from the data. Information on Nitella given by Tamiya (52) indicates $\epsilon = 32$ atm over the range of turgor changes involved in Collander's experiments. We may calculate an osmotic permeability coefficient (K_o) from the relation, which is given by Philip (41),

$$t_{½\,o} = \frac{0.693\ V_c}{AK_o(\epsilon + \Pi_o)} = \frac{0.346\ b}{K_o(\epsilon + \Pi_o)} \quad \text{XVa}$$

The formula on the right applies to a long narrow cylinder, such as the Nitella cell, for which $V/A = b/2$. The cell radius (b) is given (10) as 0.0205 cm; taking 6.5 seconds as a mean value for $t_{½\,o}$, $K_o = 2.8 \times 10^{-5}$ cm sec^{-1} atm^{-1}, and thus $K_o/K_d = 4.4 \times 10^{-2}$ atm^{-1}.

D. Thimann and Samuel (53) followed outward diffusion of tritium-labelled water (THO) from discs of potato tissue, finding half-times of approximately 1.5 minutes for discs 1 mm thick. Virgin (59) followed the progress of osmotic equilibration of strips of potato tissue 1 × 1 mm in cross section, by an ingenious resonance method, and found half-times of about three minutes. Direct comparison requires that the difference in tissue geometry be taken into account. The figures given by Philip (43) suggest that $t_{½\,d}/t_{½\,o}$ should be divided by about three to bring the two into comparison. The value of $(\epsilon + \Pi_o)$ for potato tissue can be estimated as 35 atm from data of Falk, Hertz, and Virgin (17). Whence, $K_o/K_d = 5 \times 10^{-3}$ atm^{-1}. The ratio should actually be somewhat greater, since the diffusion experiments

[5] The equation for osmotic water exchange upon which formula XV is based was derived (41) only for water absorption by a cell, initially without turgor, when placed in pure water. It is readily verified, however, that under the assumptions made in deriving it, the half-time should be the same for osmotic exchange between any initial and final water deficits of the cell not greater than Π_o.

(53) were conducted at 30° C while the osmotic experiments (59) were at room temperature.

E. Ordin and Kramer (37) found half-times of about 0.6 minutes for diffusion of DHO out of root segments of *Vicia faba*. To compare this with available data on osmotic permeability, it is necessary to compute an apparent diffusion coefficient k_d. Information given by Philip (43) indicates that for a cylindrical piece of tissue of radius b, $k_d = 0.062\ b^2/t_{½}$. Since Ordin and Kramer give the diameter of the root sections as 0.5 mm, $k_d = 6.5 \times 10^{-5}$ cm^2 min^{-1}.

Brewig (3) and Brouwer (6) measured steady osmotic water movement into *Vicia faba* root segments, in a method by which water deficit (S) difference could be determined. Their lowest values are about 0.5 mm^3 atm^{-1} hr^{-1} per cm of root length. If we assume, as was done in computing k_d above, that resistance to water flow is evenly distributed through the tissue, then the rate of water movement (v) in through each cylindrical shell of tissue, at radius b, will be

$$v = -2\pi b l k_o \cdot \frac{dS}{db} \qquad \text{XVI}$$

where l is the length of the root segment. Since the same volume of water per minute moves in through each successive cylindrical shell until the vascular tissues are reached, v can be regarded as a constant and the equation integrated between the external surface of radius b_o and the outer boundary of the stele, of radius b_i. This gives the formula

$$k_o = \frac{v}{2\pi l\ (S_i - S_o)} \ln \frac{b_o}{b_i} \qquad \text{XVII}$$

The fraction $v/(S_i - S_o)$ is the osmotic permeability, given above. From figures given by Brewig (3) the ratio of external to stele radii b_o/b_i averages 3.6. From these figures we find $k_o = 1.7 \times 10^{-6}$ cm^2 atm^{-1} min^{-1}, and the ratio $k_o/k_d = 2.6 \times 10^{-2}$ atm^{-1}.

The computed values of K_o/K_d fall in the range 4 to 44 $\times 10^{-3}$ atm^{-1}, as compared with 7.5 $\times 10^{-4}$ expected on the basis of the diffusion hypothesis. Although in all cases but the first one, osmotic and diffusion permeabilities were not measured at the same time with the same object; and while in every case there are uncertainties about some of the values used, the comparisons are nevertheless in agreement in indicating that K_o/K_d is several times larger than anticipated.

Among the possible reasons for this is the extent to which the actual situation deviates from one in which the approximations used in deriving equations XIV and XV hold good. The most serious deviation is likely to be from the assumption, made in deriving equation XV, that change in cell volume is exactly proportional to change in turgor pressure (41), since this can be far from correct (52). However, by averaging half-times for osmotic water loss and water gain this error should cancel out; this was done where possible.

It may be questioned whether permeability of membranes to DHO or THO is not less than to H_2O, which would make the measured K_d too small. The diffusion coefficients of DHO and THO are not more than 14 percent less than that of H_2O^{18}, which is believed to be approximately the same as H_2O^{16} (60). Collander (10) proved that Nitella cells are less permeable to DHO than to H_2O, because they exhibit a transient osmotic shrinkage when placed in strong DHO solutions. However, if one plots his data on osmotic transients caused by different compounds (10) against permeabilities for the same compounds, which he measured directly (11), it appears that the diffusion permeability of these cells to H_2O cannot be more than a few percent greater than to DHO. This objection has also been set aside in work with animal membranes to be discussed below.

It does not seem possible to explain with experimental errors the differences between expected and observed K_o/K_d; it seems significant that in studies on animal cells and tissues, discrepancies in K_o/K_d of similar magnitude have been found (44). Such results were reported as early as 1935 by Hevesy, Hofer, and Krogh (22). They mean that osmosis takes place too rapidly to be explained by diffusion across the membrane. Ussing (28, 56) and Pappenheimer (40) have accounted for this by concluding that osmosis is not a diffusion process, as so often assumed in plant physiology, but takes place instead by bulk flow of water through pores in the membrane. It is assumed that if the membrane has pores through which water flow can be caused by a hydrostatic pressure difference across the membrane, then under a difference of osmotic potential the membrane will behave as if an equivalent pressure difference existed across it. With artificial membranes it has been proved conclusively that osmotic water movement does occur much more rapidly than by diffusion, and as rapidly as under an equivalent hydrostatic pressure difference (31, 32). By assuming, as has been done for many years in treating flow through artificial membranes, that the pores are cylindrical and that water flow through them can be described by Poiseuille's law, average pore radii have been calculated, from K_o and K_d values, for several animal membranes, in careful work by Solomon and collaborators (16, 38, 49, 58). It is interesting to note that as long ago as 1930 Huber and Höfler (25), who considered osmotic water movement through plant membranes as a flow rather than a diffusion, attempted to calculate radii for pores in the Salvinia cell membrane by using a formula which had been worked out for artificial membranes.

Cause of Flow Through Membranes. It is not obvious that a concentration difference across a membrane should have the same effect as a pressure difference, and thus lead to flow rather than diffusion of water through pores in it. Chinard (7, 8) has objected to the concept of water flow, pointing out

that in the presence of a concentration difference alone, no hydrostatic pressure difference capable of causing a flow exists across the membrane. Dick (14) and Harris (21) also reject the idea of bulk flow through cell membranes, stating that Poiseuille's law should not hold in channels of molecular dimensions.[6] No physical explanation of why water flow should occur under a concentration difference seems to have become widely accepted. This section presents a proposal about the mechanism of osmotic water movement from which, it is felt, the occurrence of water flow through membranes having pores can be seen to be natural and inevitable.

We shall consider, as shown in figure 3a, a membrane of thickness l, containing pores of radius b, which allow the entry of water but not of osmotically active solutes present in the adjacent solutions. If, first, pure water is present on both sides of the membrane, and isotopically labelled water is introduced on one side, it will diffuse through the membrane along a concentration gradient $(c_1 - c_2)/l$. If, however, an osmotic solution is placed on one side (c_2) and pure water at the same pressure on the other side (c_1), it is seen that the concentration gradient is, effectively, restricted to the aperture of the pore opening into c_2, since only water, not solute, can pass into the pore. The distance over which the concentration changes from c_2 to pure water will be about the same as the pore radius b, since solute molecules which are, for example, larger than the pore aperture will not be able to penetrate any farther into the pore. Thus, for a given difference in water concentration induced osmotically, the concentration gradient will be steeper, and diffusion will tend to be more rapid, than for labelled water in proportion as l is larger than b. If the pore presented little resistance to water flow, water would flow into and through the pore from c_1 as fast as it diffuses out along the concentration gradient into c_2 at the pore aperture, so causing osmotic water movement to take place by bulk flow and to be more rapid than can be accounted for by measuring diffusion of water across the membrane.

However, with thick artificial membranes, and probably with natural membranes as well, the relation between b and l is such that considerable viscous resistance to water flow is offered by the pore. Rather than preventing water flow, this proves to be the feature by which pressure effects and osmotic effects become closely comparable, as we shall now see. Diffusion of water from within the pore aperture into c_2, if not compensated for by flow from c_1, will result in a decrease in the volume of water within the part of the pore adjacent to the aperture, and hence a decrease in pressure there. Thus there arises

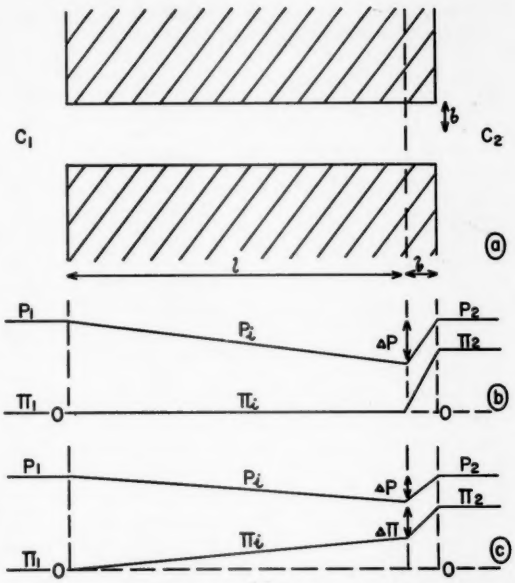

FIG. 3. Osmotic behavior of pores in a membrane. a: Diagram of the pore. b: Changes in solute osmotic potential Π and pressure P along the length of the pore, if membrane is impermeable to solute and separates a solution (at c_2) from water (at c_1), the solution and water being at the same pressure. c: Similar changes for a membrane permeable to the solute.

a pressure gradient within the pore, between solution c_1 and the aperture of the pore next to solution c_2, along which water must flow (and diffuse) from c_1 to c_2. This situation is illustrated in figure 3b.

Diffusion out of the pore aperture into c_2 can be described by equation XI or, more simply, with its approximate form, equation XIV. If we call ΔP the pressure drop within the pore aperture and remember that there is only water within the pore, then the rate of diffusion of water through the aperture will be

$$v = \frac{A k'_w \overline{V}}{RTb} (\Pi_2 - \Delta P) \qquad \text{XVIII}$$

where A represents the cross-sectional area of the pore, and k'_w the diffusion coefficient of water in the pore. Considering now water movement within the pore as the sum of diffusion and bulk flow under the pressure gradient $\Delta P/l$, then if flow is described by Poiseuille's law

$$v = \frac{A k'_w \overline{V} \cdot \Delta P}{RTl} + \frac{A b^2 \cdot \Delta P}{8 \eta l} \qquad \text{XIX}$$

During steady osmosis, ΔP must attain such a value that the rate of water movement through the pore is equal to the rate of movement out of the pore aperture

[6] Harris (21) offers an explanation for the discrepancy between measured rates of osmosis and water diffusion which is, actually, a proposal that bulk flow through pores is driven by differences in molecular bombardment at the two ends of the pore.

into c_2. Hence we may set equations XVIII and XIX equal to one another and solve for ΔP.

$$\Delta P = \frac{\Pi_2}{1 + \frac{b}{l} + \frac{RTb^3}{8\eta k'_w \bar{V} l}} \qquad XX$$

This indicates that the pressure drop within the pore will approach the numerical value of the osmotic potential difference between the external solution and that inside the pore, provided l is sufficiently larger than b. This is equivalent to saying that under these conditions, diffusion equilibrium will practically prevail between the water in solution c_2 and the contents of the pore just inside the aperture, and that osmotic water movement is controlled by the rate of water flow within the pore. The range of pore dimensions within which this will be the case can be seen by computing the value of the denominator in equation XX for arbitrary values of b and l, which has been done in table I. Whenever this value is not significantly greater than one, the pressure drop within the pore will approach the osmotic potential of the external solution c_2.

If diffusion equilibrium practically prevails at the pore aperture next to c_2, it should do so also at the aperture next to c_1, so if c_1 is now an osmotic solution, the pressure drop induced within the length of the pore should be equal to the difference in pressure drops ΔP which arise at its two apertures, or equal to $\Pi_2 - \Pi_1$. It thus becomes apparent why an osmotic potential difference should cause the membrane to behave as if a comparable hydrostatic pressure difference existed across it. The rate of water movement through the pore will be equal to the water flow and diffusion induced along its length by the total pressure difference between its ends, this being the pressure difference induced osmotically plus any hydrostatic pressure difference between the two solutions, so from equation XIX,

$$v_{1\to 2} = \frac{A}{l}\left[\frac{k'_w \bar{V}}{RT} + \frac{b^2}{8\eta}\right]\left[(\Pi_2 - \Pi_1) - (P_2 - P_1)\right] \qquad XXI$$

This has the same form as equation XIV, but a larger value because of the appearance of the bulk flow term $b^2/8\eta$. As shown by Pappenheimer (40), this term becomes less and less important as the pore radius is reduced, until at very small radii osmotic water movement would have the appearance of pure diffusion; it is to be noted, however, that within the membrane this diffusion is considered to be occurring along a pressure gradient rather than a concentration gradient. On the other hand, at radii where the bulk flow term is large compared with the diffusion term, flow is the principal mechanism of osmotic water movement, and osmosis occurs more rapidly than diffusion of water across the membrane, as in the examples examined in the preceding section.

For membranes in which the pressure drop ΔP induced osmotically is less than the osmotic potential difference $\Pi_2 - \Pi_1$ (the denominator in equation XX being greater than one), it appears that the rate of osmotic water movement must be less than the water flow under a numerically equal hydrostatic pressure difference, so that equation V would not hold true. The limiting case, where ΔP approaches zero, was the case discussed initially in this section. Actually, the pore dimensions in which this situation could arise (table I) are mostly such as to make the membrane rather or very permeable to dissolved substances, a situation which would also decrease osmotic flow in comparison with pressure flow, as will now be discussed.

DIFFUSIBLE SOLUTES. The restricted diffusion permeability of a porous membrane to a solute[7], compared to water, may be considered as the result of two effects:

I. Within the pores of the membrane, the diffusion coefficient of the solute (k'_s) is restricted, due to frictional hindrance to molecular movement, to a greater extent than is that of water (k'_w).

II. At the pore aperture entry of solute molecules is restricted, by comparison with water molecules, because molecules which impinge upon the pore aperture can enter the pore only if they are not deflected by striking its edge: for the larger solute molecules, a smaller fraction of the total pore area can be hit without involving collision with an edge, than for water molecules. This geometrical effect is discussed by Pappenheimer (40). That fraction of the pore area available to water, which is not available to solute in consequence of this effect, will be called the reflection factor ϕ for the solute. Its maximum value is one, in the case of a membrane completely impermeable to the solute, as in the preceding section.

Diffusional entry of molecules into the pore will be in proportion to the number of collisions with the

[7] The solutes referred to are, throughout, lipid-insoluble ones incapable of penetrating the membrane at an appreciable rate by solution in a lipid phase.

TABLE I

RATIOS OF Π TO ΔP FOR VARIOUS PORE DIMENSIONS*

PORE RADIUS, Å	PORE LENGTH, Å			
	10	100	1,000	10,000
3	1.5	1.05	1.0	1.0
10	9.2	1.8	1.08	1.01
30	199	21	3.0	1.2
100	7,200	721	73	8.2

* Calculated from equation XX using the viscosity and diffusion coefficient (60) of water at 25° C.

pore aperture which do not result in reflection at the edges. Effective collisions of solute and of water molecules will be in proportion to their mole fractions X_s and X_w, and to the relative pore areas available to them ($[1 - \phi]$ to one). Hence during steady diffusion of solute from a solution to pure water across the membrane, the mole fractions of solute and water just inside the pore aperture (X'_s, X'_w) will be related to those outside by the expression

$$\frac{X'_s}{X'_w} = (1 - \phi)\frac{X_s}{X_w} \qquad \text{XXII}$$

The resulting solute mole fraction profile is diagrammed in figure 3c.

For ordinary solutions we may, as before, with sufficient accuracy take X_w and X'_w equal to one. Thus the difference in solute mole fraction outside and inside the aperture ($X_s - X'_s$) will be ϕX_s. Water will diffuse towards solution c_2 along the resulting concentration gradient at the pore aperture, and, for the reasons given in the previous section, if the pore presents sufficient resistance to flow of solution, the pressure inside the aperture will fall until it differs from the pressure at c_2 by the difference in osmotic potential; this difference is seen to be $\phi\Pi_2$. Figure 3c shows the resultant pressure profile within the pore. Along the pressure gradient inside the pore a bulk flow of solution towards c_2 will take place. As before, we shall assume this flow to be described by Poiseuille's law.

The net volume movement from solution c_1 (pure water) to solution c_2 will be the sum of the forward flow of solution along the pressure gradient within the pore and the diffusion of water along the gradient of pressure and concentration within the pore, less the back diffusion of solute along its concentration gradient inside the pore:

$$v_{1\to 2} = \frac{A}{l}\left\{\frac{b^2}{8\eta}(P_1 - P_2 + \Delta P) + \frac{k'_w\overline{V}}{RT}(P_1 - P_2 + \Delta P) + k'_wX'_s - k'_sX'_s\right\} \qquad \text{XXIII}$$

Equation XXIII neglects the effect of pressure on diffusion of solute, which will be small compared with the effect on water because the mole fraction of solute is small, and it treats the diffusion of water and solute as independent of flow of solution. This approximation is satisfactory as long as the solute concentration difference is sufficiently small in relation to the hydraulic conductivity of the membrane, as can be seen from the equations given by Jacobs (27). It appears that this condition holds sufficiently well for biological situations and membranes with small pores, such as cell membranes, which are of interest here. Otherwise, the relationship between osmotic flow and solute concentration becomes more complex. The value of η to be used in equation XXIII and those following, is that for the average composition of the solution flowing within the pore.

Substitution, for ΔP and X'_s, of the values given above, leads to

$$v_{1\to 2} = \frac{A}{l}\left\{\left[\phi\left(\frac{b^2}{8\eta} + \frac{k'_w\overline{V}}{RT}\right) + (1 - \phi)\frac{(k'_w - k'_s)\overline{V}}{RT}\right]\Pi_2 - \left[\frac{b^2}{8\eta} + \frac{k'_w\overline{V}}{RT}\right](P_2 - P_1)\right\} \qquad \text{XXIV}$$

To obtain volume equilibrium between solutions 2 and 1 it would be necessary to impose such a pressure difference (P_2–P_1) that $v = 0$. This required pressure is called the "effective osmotic pressure" of solution c_2, and from equation XXIV it is seen to be

$$(P_2\text{–}P_1)_0 = \left[\phi + (1 - \phi)\frac{(k'_w - k'_s)\overline{V}/RT}{\frac{b^2}{8\eta} + \frac{k'_w\overline{V}}{RT}}\right]\Pi_2 = \chi\Pi_2 \qquad \text{XXV}$$

The coefficient χ will be called the "osmotic effectiveness" of the solute being considered. It varies from zero, for a solute lacking any osmotic effect, to one, for a solute to which the membrane is completely impermeable. Substitution into equation XXIV gives

$$v_{1\to 2} = \frac{A}{l}\left(\frac{b^2}{8\eta} + \frac{k'_w\overline{V}}{RT}\right)\left(\chi\Pi_2 - [P_2\text{–}P_1]\right) \qquad \text{XXVI}$$

It can be seen that the osmotic flow induced by a diffusible solute should be governed by the effective osmotic pressure of the solution $\chi\Pi_2$ rather than by its osmotic potential Π_2. This fact was deduced on thermodynamic grounds by Staverman (50, 51). Meschia and Setnikar (32) recently showed that osmosis across a collodion membrane, with a series of solutes to which the membrane differed in permeability, obeys an equation of this type. The general expression for osmotic movement, developed as above

but considering the effect of different solutes, and of solutes on both sides of the membrane, is

$$v_{1\to 2} = \frac{A}{l}\left[\frac{b^2}{8\eta} + \frac{k'_w\overline{V}}{RT}\right]\left[\sum_s \chi_s\,(\Pi_{s,2}-\Pi_{s,1}) - (P_2-P_1)\right] \quad \text{XXVII}$$

It is clear that in the presence of solutes to which the membrane is permeable, osmosis should not follow equation V. This has important consequences. It is not correct to treat osmotic flow induced by diffusible solutes as proportional to water deficit difference, as has been done [e.g., (41)]; the osmotic effectiveness χ of the solutes must be included. Moreover, equation V fails in that most important aspect of water movement in plants, transport through the xylem, for the cell walls of xylem elements are considerably permeable to solutes. Evidence showing that equation V cannot be applied to the xylem was obtained, for example, by Jaccard and Frey (26), who measured osmotic permeability (with sucrose) and hydraulic conductivity of several wood membranes. Their data show that the latter conductivity exceeded the former by a large factor in all cases; this was associated with substantial permeability to sucrose. Therefore, it is feasible to include water movement in the xylem in equation VI only if differences in solute concentration within it are quite small.

According to the present considerations, the relationship between χ and the diffusion permeability of the membrane to water and solute will not in general be a simple one. Since diffusion permeabilities to water (K_d) and solute (K_s) are proportional to k'_w and $k'_s(1-\phi)$, respectively, it can be shown from equation XXV that for a diffusion membrane (bulk flow term small in comparison with diffusion term), χ reduces to $1 - K_s/K_d$. For a membrane through which movement by bulk flow predominates under a pressure difference (bulk flow term large compared with diffusion term), χ is essentially equal to ϕ except at small values of ϕ, or to $1 - K_s k'_w/K_d k'_s$, so that osmotic effectiveness should be less than $1 - K_s/K_d$. According to the theory of Renkin (46), with small or moderate restriction of permeability, the contributions of restriction at the pore aperture and to diffusion within the pore should be of comparable magnitude. From equation XXV this would lead to the relationship

$$\chi = 1 - \left(\frac{K_s k'_w}{K_d k_s}\right)^{1/2}\left(1 + \frac{\overline{V}(K_d-K_s)}{RTH}\right) \quad \text{XXVIII}$$

where k_s is the free diffusion coefficient of the solute, and H is the hydraulic conductivity of the membrane. At moderately restricted permeability to solute, the second term in brackets may be neglected for flow membranes. If restriction to diffusion of water within the pores is only slight, the first term in brackets could be evaluated using measured permeabilities and known free diffusion coefficients of water and solute. At very slight restrictions to diffusion of solute, this term becomes practically equal to one; then the principal contribution to χ is made by the last term of equation XXVIII, or the $(1-\phi)$ term of equation XXV, which specifies the pressure required to cause a bulk flow just sufficient to offset the difference in diffusion rates of water and solute through the membrane. This represents the situation in which virtually no bulk flow is caused by the solute concentration difference, and a weak, purely diffusional osmotic movement can occur. This seems to have been the case in experiments with diffusible solutes by Meschia and Setnikar (32), for $K_s k_w/K_d k_s$ was very nearly equal to one with glucose, sucrose, and raffinose [cf. diffusion coefficients given in (46)], and the observed χ can be calculated fairly closely using the last term in equation XXVIII. These authors (32) employed the hydraulic conductivity of the membrane to calculate χ from rate of osmotic movement observed under a diffusible solute concentration difference, which gave an appearance of occurrence of bulk flow which we suggest was a purely formal one, and in reality pertained to the bulk flow which occurred when a pressure difference was imposed to maintain volume equilibrium. In any event, the experiments show clearly that χ was much smaller than $1 - K_s/K_d$, which is predicted for flow membranes by the present hypothesis of the mechanism of osmosis, but might not have been expected intuitively [cf. (40)].

Meschia and Setnikar's (32) permeability data are given on the conventional concentration basis, rather than on the mole-fraction basis adopted here to simplify the treatment of pressure effects. Osmotic movement can be derived, using diffusion coefficients on a concentration basis, in a fashion similar to that employed above, yielding an expression for χ slightly more complex than equation XXV. It seems likely that the real relationship should lie somewhere in between mole-fraction and concentration basis. In evaluating Meschia and Setnikar's data the concentration basis was adhered to.

The above interpretation of osmotic water movement is not basically dependent upon any of the simplifying assumptions made about the shape or path of the pores, or the applicability of particular diffusion coefficients or of Poiseuille's law to movement within pores. As has frequently been remarked, it is unlikely that Poiseuille's law in unmodified form can accurately describe bulk flow through channels of molecular dimensions; which is not to say that bulk flow cannot occur, as has sometimes been implied, but that the term $b^2/8\eta$ in the equations should be replaced by some other term dependent on b and η. Such departures from the simple assumptions used here are equivalent to having channels in the membrane related to b and l in the equations and in table I by some numerical factor. Furthermore, consider-

ing the nature of processes at molecular dimensions, the pressure and concentration gradients expected within the pores should constitute a time- or number-average of the situation, rather than the invariable state of every pore. Finally, the basic osmotic equation XXVII cannot be expected to hold except for dilute solutions and low pressures, as it contains the approximations which are the basis for equation XIV, and depends additionally upon pore geometry.

One point which has been omitted entirely is the possibility of a backflow from solution c_2 into the pore aperture along the pressure gradient expected to develop in the aperture of the pore. A flow from solution into pore aperture would be an ultrafiltration rather than a simple flow, since for a membrane not permeable to solute, only water would pass. Such a flow would involve viscous resistance between solute molecules and water as well as between water and the pore, and hence must be slow by comparison with water flow within the pore. In any case, the supposed flow would not be possible thermodynamically unless the pressure drop in the pore aperture *exceeded* the expected ΔP (18).

Regardless of these questions, the important point appears to be that osmotic flow of water through membranes can be viewed as the consequence of pressure gradients set up within the pores by rapid diffusion at the pore apertures, across the sharp concentration gradients prevailing there. Even the idea of discrete pores, useful in quantitative treatment, is not essential to the occurrence of osmotic flow, the only requirements being that water permeate the membrane as a continuous phase while entry of solutes be restricted at the membrane surfaces. An intuitive treatment of osmotic water movement based on this picture of membrane structure was presented as long ago as 1935 by Jacobs (*27*). The writer's attention has lately been called to the discussion of osmosis given by Ussing and Andersen (*57*), who depict a porous membrane as similar to figure 3a, and regard the cause of bulk flow to be lack of penetration of solute into the pore, as was arrived at independently in the present study.

Summary

The mechanism of osmotic water movement and the factors which determine its rate are reexamined from a quantitative point of view. Diffusion pressure and diffusion pressure deficit are held to be self-contradictory concepts, and hence not tenable. Suction force, which is an acceptable concept relating to states of osmotic equilibrium, is renamed "water deficit" to avoid undesirable implications of the former term which have previously been criticized. Rate of water movement cannot be proportional to differences in water deficit as a general law, contrary to the usual assumption. The idea that transpiration rate is proportional to the suction force of atmospheric water vapor is completely erroneous. However, on the assumption that osmosis is due to diffusion across membranes, proportionality between its rate and differences in water deficit is an approximation good for low pressures and dilute solutions. The osmotic permeability of a membrane is then expected to be related to the diffusion permeability to water, as measured with labelled water, by the factor $\overline{V}/RT$. Available measurements on plant cells and tissues indicate that osmotic permeability exceeds that expected on the basis of diffusion by a factor of at least several fold, which cannot be explained by experimental errors. It thus appears, as previously observed with animal membranes, that osmosis is not strictly a diffusion process. The idea is examined that osmosis occurs by bulk flow of water through pores in the membrane. Occurrence of flow through pores appears to be an inevitable result of pressure gradients induced within the pores by diffusion at pore apertures. This explains why an osmotic potential difference across a membrane causes as fast a water flow as a numerically equal hydrostatic pressure difference. The hypothesis can also be applied to osmosis induced by diffusible solutes, which is not proportional to differences in water deficit.

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List of Symbols

A Area (cm^2) of membrane, or of pore cross section

b Radius (cm)

c Concentration (moles $liter^{-1}$)

d_1 Rate of outward diffusional movement of water molecules, from solution 1, through a membrane (cm^3 sec^{-1})

H Hydraulic conductivity of membrane (cm sec^{-1} atm^{-1})

K_d Diffusion permeability of membrane to water, equal to k_d/l (cm sec^{-1})

K_0 Osmotic permeability of membrane, equal to k_0/l (cm sec^{-1} atm^{-1})

K_s Permeability of membrane to a solute (cm sec^{-1})

k_d Diffusion coefficient of water across membrane, mole fraction basis (cm^2 sec^{-1})

k_0 Osmotic permeability coefficient (cm^2 sec^{-1} atm^{-1})

k'_s Restricted diffusion coefficient of solute within pore (mole fraction basis, cm^2 sec^{-1}) (k_s is the free diffusion coefficient)

k'_w Restricted diffusion coefficient of water within pore (mole fraction basis, cm^2 sec^{-1}) (k_w is the free diffusion coefficient)

l Membrane thickness (cm); length (cm) in equations XVI and XVII

P Hydrostatic pressure, in atm greater (+) or less than (−) atmospheric.

ΔP Pressure difference between external solution and contents of pore just inside aperture (atm)

p Partial vapor pressure of water (atm)

R Gas constant (82.0 cm^3 atm deg^{-1} $mole^{-1}$)

r_{21} Resistance to flow between points 1 and 2 (atm sec cm^{-3})

S Water deficit (suction force), defined by equation I (atm)

S′ Suction force of atmospheric water vapor (atm), defined in the section on transpiration.

T Absolute temperature (deg K)

$t_{½}$ Time for process to proceed halfway from initial state toward completion. Subscripts o and d refer to half-times for osmotic equilibration and diffusion exchange of labelled water (external concentration constant), respectively.

$\bar{V}$ Partial molal volume of water (cm^3 $mole^{-1}$)

V_c Cell volume (cm^3)

$\nu_{1\to2}$ Rate of volume movement from unit 1 to unit 2 (cm^3 sec^{-1})

X Mole fraction of water in a solution, in osmolar units.

X_s Mole fraction of solute, in osmolar units.

X'_s Mole fraction of solute inside pore aperture.

ϵ Elastic modulus of the cell wall (atm), defined as in (41)

η Viscosity (atm sec). The usual unit of viscosity is the poise (dyne cm^{-2} sec), with which pressure must be expressed in dynes cm^{-2}. Since it is customary and convenient in plant physiology to express pressure in atmospheres, viscosity as used here is in atm sec, which is equal to viscosity in poises multiplied by 9.87×10^{-7}.

Π Osmotic potential (atm)

Π_0 Osmotic potential at zero turgor (atm)

$\Pi_{s,1}$ Contribution of a particular solute to the osmotic potential of solution 1 (subscript$_{s,2}$ is the same for solution 2) (atm)

ϕ Reflection factor for a solute, defined in the section on diffusible solutes.

χ Osmotic effectiveness of a solute, defined so that $\chi\Pi$ is the pressure required to effect volume equilibrium between the solution and water through a particular membrane.

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OXIDATIVE ACTIVITY OF CYTOPLASMIC PARTICLES OF APPLES: ELECTRON TRANSFER CHAIN

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Some recent studies of apple respiration by Hackney (8) implied that the fruit may mediate its oxidations through a phenol oxidase system. In a later study Webster (21, 22) could find no evidence for a cytochrome oxidase system in apple tissue. However, more recently a number of reports have appeared which demonstrated cytochrome oxidase activity in apple tissue slices (14) and in cytoplasmic particles from apples (13, 16). There are also data showing Krebs cycle activity in cytoplasmic particles from apples (9, 18). Nevertheless, a number of workers are still experiencing considerable difficulty in isolating active cytoplasmic particles from apples (15). The difficulties might be traced to characteristics of the apple fruit, such as a highly acid cell sap (pH 3.5), an extremely low concentration of protein (approximately 0.3 %), and a high concentration of polyphenol substances that may injure cytoplasmic particles during homogenization. As a first step in isolating active cytoplasmic particles from such tissues it seemed advisable, as previously noted (13), to investigate the effect of homogenization in media of increasing pH.

This paper reports the results of such a study. Data are presented to show that alkaline homogenates yield the most active cytoplasmic particles. The properties of the particles from homogenates of different pH are described in some detail and spectra are presented demonstrating the presence of cytochrome b and cytochrome a_3 in the particles. These data and additional evidence from inhibitor studies lead to the conclusion that the apple particles isolated from alkaline homogenates contain the usual type of electron transfer chain.

MATERIALS AND METHODS

Malus sylvestris (Miller) Rome Beauty apples harvested during the first week of October, were used in these experiments. The fruit held in cold storage (0° C) and used during the storage season from October to May.

Three hundred grams of apple pulp were grated into a blendor cup containing 300 ml of cold 0.25 M sucrose, 0.2 M buffer (phosphate, or tris(hydroxymethyl) amino methane (tris), or sodium carbonate), 0.01 M ethylene-diamine-tetra-acetic acid (EDTA) and 0.01 M ascorbic acid. Homogenization was carried out for 2 minutes at reduced speed (approx 40 v) controlled with a variac. The homogenates were strained through four layers of cheesecloth and centrifuged for 10 minutes at approximately 1,000 × G followed by a high speed centrifugation of the supernatant at about 17,000 × G for 15 minutes. The particles obtained from the high speed centrifugation were washed in 0.25 M sucrose and resedimented at 17,000 × G. The particles were finally suspended in 6 ml of 0.25 M sucrose and 0.001 M adenosine triphosphate (ATP) adjusted to pH 7.0. These suspensions generally had a nitrogen (N) content (N of ATP subtracted) that varied from 250 to 900 μg/ml depending on the buffer of the homogenizing medium. All operations were carried out in a cold room held at 2 to 4° C.

ASSAY PROCEDURES: Spectrophotometric assays were conducted at room temperature (approx 20° C) with a Beckman DU spectrophotometer in cuvettes of 1 cm path length and 3 ml volume. Readings were taken every 30 seconds for 3 minutes after adding the enzyme, DPNH, or cytochrome c at zero time to start the reactions. All rates given are corrected for endogenous activity as determined from blanks.

Cytochrome oxidase activity was determined by following the decrease in optical density at 550 mμ after adding the apple particles to reducted cytochrome c (Sigma), according to the method of Cooperstein and Lazarow (4). The reaction mixture contained 2.98 ml of reduced cytochrome c (0.076 μ moles) in 0.03 M phosphate buffer pH 7.1, and 0.02 ml of the particles added at zero time. The oxidation of reduced cytochrome c was found to be linear for at least 1 minute and was proportional to enzyme concentration from 0.01 to 0.04 ml of the particulate suspension. This assay was run against a water blank.

The oxidation of reduced diphosphopyridine nucleotide (DPNH) (Pabst) was determined by following the rate of decrease in optical density at 340 mμ after adding 0.05 ml of the particulate suspension at zero time, to a cuvette containing 60 μ moles of tris buffer pH 7.4, 0.15 μ moles DPNH, 0.03 μ moles cytochrome c, and water to bring the volume to 3 ml. The readings were compared to a blank containing all the components except for the absence of DPNH.

DPNH—Cytochrome c reductase activity was assayed by determining the increase in optical density at 550 mμ in the above system except that 0.06 μ moles of cytochrome c was used and the oxidation of reduced cytochrome c was blocked with 8 μ moles of sodium

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azide. The assay was run against a blank containing all the components except DPNH.

Diaphorase activity was determined by following the reduction of 2,6,dichlorophenol-indophenol (DCPIP) as a decrease in optical density at 600 mμ, in a system containing the same components as the DPNH—cytochrome c reductase assay except that 0.15 μ moles of DCPIP was used instead of cytochrome c. The assay was run against a blank containing all the above additions except DPNH.

Succinic—cytochrome c reductase activity was assayed by following the increase in optical density at 550 mμ after incubating the following mixture for 10 minutes at 25° C: 60 μ moles phosphate pH 7.0, 6 μ moles succinate, 8 μ moles sodium azide, 3 μ moles $MgCl_2$, 0.05 ml of the particulate suspension and water to a volume of 2.9 ml. The reaction was started by adding 0.06 μ moles of cytochrome c to the incubated mixtures. The blanks were treated exactly the same except that they contained no succinate.

Succinoxidase was determined by standard Warburg manometric methods (20). The vessels contained in addition to 30 μ moles succinate, 1 μ mole adenosine triphosphate (ATP), 12 μ moles of magnesium, 20 μ moles phosphate, 0.1 ml of 1 % yeast concentrate (Sigma), 0.3 ml 2 % bovine serum albumin, 0.001 μ moles cytochrome c, 1.0 ml of the particulate suspension, and 0.25 M sucrose to a volume of 3 ml. All the additions were buffered to pH 7.1.

Manometric techniques were also used to determine the carbon monoxide inhibition of cytochrome oxidase and its reversal by light. The vessels contained 0.06 μ moles cytochrome c (Sigma), 40 μ moles phosphate buffer pH 7.1, 2 μ moles ATP, 12 μ moles Mg, 0.5 ml of the particulate suspension, 30 μ moles ascorbic acid, freshly prepared and adjusted to pH 7.1, and 0.25 M sucrose to a volume of 3.0 ml. The ascorbic acid, used to reduce cytochrome c, was placed in the side arms and tipped into the main compartment of the vessel at zero time, after 10 minutes of equilibration to temperature (25° C). Darkness was achieved by completely covering the bath and vessels with a double layer of black cloth. Light was provided by two 150 w G.E. photoflood lamps (PAR 38) hung about eight inches from the surface of the water bath. These lamps emit about 3.5 per cent of their energy in the 350 to 700 mμ region of the spectrum.

Nitrogen determinations were made by the Thompson and Morrison method (19).

The data reported are the averages of at least three experiments.

Additional experimental details are described in the text and legends of the tables and figures.

Results

Most of the difficulties encountered in isolating active cytoplasmic particles from apple fruit appear to be related to the presence in the cells of high concentrations of potentially inhibitory substances, notably polyphenols (10, 15), and to the acidity of the cell sap (17). The effects of polyphenols were considerably reduced by eliminating the peel, which contains the bulk of these substances, and by introducing 0.01 M ascorbic acid into the homogenizing medium, thus maintaining the polyphenols in the reduced (non-inhibitory) state (12). Having established what appeared to be a satisfactory operating procedure with respect to avoiding inhibition by phenolics, the effects of polyphenols were not further considered in these studies. However, a detailed analysis was made of the effects of homogenizing media of increasing alkalinity on the activity of cytoplasmic particles isolated from them.

pH of Homogenates and Activity of Particles: The acidity of the cell sap was counteracted by using strong alkaline buffers in the homogenizing media. Table I lists the types of buffers, the concentrations

Table I

pH of Homogenates and Particulate Suspensions Derived From Homogenizing Solutions Containing Buffers of Increasing Alkalinity

Buffer*	pH of Buffer	pH of Homogenate	pH of Particulate suspension**	mg N/ml Particulate suspension***
0.1 M K_2HPO_4 0.1 M KH_2PO_4	6.7	6.0	6.3	0.90
0.2 M K_2HPO_4	8.1	6.9	6.6	0.90
0.2 M Tris	9.2	8.4	7.2	0.55
0.1 M Na_2CO_3	10.2	9.3	7.1	0.50
0.2 M Na_2CO_3	10.6	9.9	7.6	0.30
0.2 M Na_2CO_3†	12.0	10.7	8.5	0.25

* In addition to buffer all the homogenizing media contained 0.25 M sucrose, 0.01 M EDTA, and 0.01 M ascorbate. Three hundred grams of tissue were blended with 300 ml of homogenizing solution as described in the text. Distilled water homogenates of apples had a pH of 3.4.

** All particles were washed once in 0.25 M sucrose and finally suspended in 6 ml of 0.25 M sucrose and 0.001 M ATP at pH 7.0.

*** The N of the suspending medium was subtracted.

† This medium was made to pH 12.0 with solid KOH.

used, and the pH's of the resultant homogenates, under the conditions indicated in the legend. Cytoplasmic particles isolated from phosphate buffers which gave homogenates near neutrality showed little or no electron transport activity (table II). Thus, buffering the homogenate to yield a suspension near neutrality, a condition satisfactory for many plant tissues, was completely inadequate with apple fruit tissue. Particles isolated from alkaline buffers, however, yielding alkaline homogenates above pH 8 manifested significant electron transport chain activity. Maximum activity of DPNH oxidase, diaphorase, DPNH—cytochrome c reductase, and cytochrome oxidase was observed in cytoplasmic particles isolated from homogenates of pH 9.9 (table II). It should be borne in mind that the pH of the particulate suspension was 7.6 (table I).

Cytochrome Oxidase Activity and Inhibition: The cytochrome oxidase activity of the particles isolated from 0.2 M sodium carbonate-sucrose medium (pH of homogenate 9.9 of table II), in a system in which cytochrome c was reduced by ascorbic acid (fig 1). The activity was stable for approximately two hours. The inclusion of an inhibitor of polyphenolase, as reported by Rubin and Ladygina (16) was not found necessary in these systems, even though the particles are known to contain polyphenolase. It is interesting to note, in addition, that the cytochrome oxidase system in these preparations was stable for at least 3 weeks at 5° C.

Carbon monoxide inhibition and reversal by light was demonstrated in these preparations (fig 1). It was not possible, however, to incorporate inorganic phosphorus into these systems even when supplied with hexokinose and glucose in addition to the other components of the system (ATP, Mg, phosphate). This indicates that the phosphorylation mechanism,

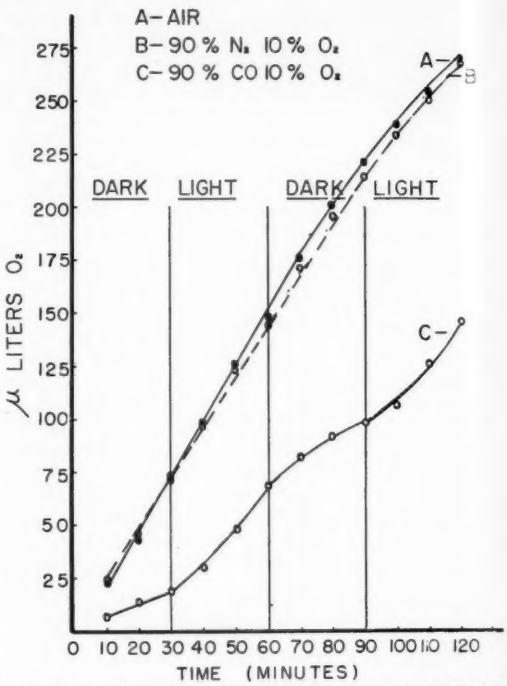

Fig. 1. Light reversal of carbon monoxide inhibition of cytochrome oxidase activity. The reactions were run at 25° C. in a Warburg apparatus. Each of the appropriate vessels was flushed with ten volumes of either N_2/O_2 mixtures in proportions of 9:1 or CO/O_2 in proportions of 9:1 before closing the system and equilibrating to temperature. Details of the reaction mixtures and other procedures are described in the text.

Table II

Activity of Electron Transport Chain Components of Cytoplasmic Particles Isolated From Homogenates of Different pH*

pH of Homogenate**	Specific activity			
	DPNH Oxidase***	Diaphorase†	DPNH-Cytochrome c reductase††	Cytochrome oxidase‡
6.0	0.005	0.006	0.000	0.000
6.9	0.011	0.012	0.003	0.000
8.4	0.025	0.041	0.022	0.100
9.3	0.081	0.067	0.072	0.630
9.9	0.153	0.079	0.204	1.120
10.7	0.047	0.035	0.026	0.150

* Details of assay procedures are described in the text. The rates are calculated from the absorption coefficients (5).

** Homogenates are those described in table I.

*** μM DPNH oxidized/min/mg N.

† μM 2,6-dichlorophenolindophenol reduced/min/mg N.

†† μM cytochrome c reduced/min/mg N.

‡ μM cytochrome c oxidized/min/mg N.

TABLE III

SUCCINATE OXIDATION BY CYTOPLASMIC PARTICLES ISOLATED FROM HOMOGENATES OF DIFFERENT pH*

pH OF HOMOGENATE**	SUCCINIC-*** CYTOCHROME C REDUCTASE (μM CYT. C REDUCED/MIN/MG N)	SUCCINIC-† OXIDASE (μ MOLES SUCCINATE OXIDIZED/MIN/MG N)
6.0	0.000	...
6.9	0.002	0.092
8.4	0.088	0.446
9.3	0.041	0.288
9.9	0.020	0.180
10.7	0.000	...

* Details of assay procedures are described in text.
** Homogenates are described in table I.
*** Calculated from spectrophotometric assay.
† Calculated from manometric assay.

which is usually assumed to be a more labile system in mitochondria, was damaged and that electron transfer was proceeding in an uncoupled manner.

OXIDATION OF SUCCINATE: In contrast to the oxidation of electron transport chain components, DPNH, and reduced cytochrome c, which showed maximal activity with particles isolated from homogenates of pH 9.9, the oxidation of succinate was maximal with cytoplasmic particles derived from homogenates of pH 8.4 (table III). This was true for both succinic-cytochrome c reductase and succinoxidase activity. As a matter of fact there was no demonstrable succinate activity in particles from homogenates of pH 6.0, and the activity of particles from pH 9.9 was much greater than that from pH 6.9.

These data offer evidence for the necessity of an alkaline homogenizing medium to isolate active cytoplasmic particles from apple fruit. It is further evident that a more alkaline homogenate (pH 9.9) yields particles with better DPNH cytochrome c reductase, DPNH oxidase, and cytochrome oxidase activity while homogenates of lower pH (8.4) furnish particles with better succinoxidase activity. In other data (unpubl), the oxidation of other Krebs cycle substrates was also favored by particles from homogenates of pH 8.4 compared to pH 9.9.

EFFECT OF INHIBITORS: The effects of antimycin A and amytal, reported to be inhibitors of specific sites in the electron transport chain (2), are shown in table IV. In these studies the particles were prepared in the alkaline homogenates that yielded the best activities as described above. More than 75 % of DPNH oxidase, DPNH-cytochrome c reductase and succinic-cytochrome c reductase were inhibited by antimycin A (0.5 μg per cuvette) (table IV). Inhibition of the succinoxidase system declined with time from 66 % to 21 % after 2 hours. This may indicate that electron transport from succinate to oxygen proceeds, under these conditions, via a pathway which circumvents the antimycin A block. Alternatively the decline in inhibition of the succinoxidase system may result from alteration or destruction of the antimycin A with time.

The inhibitory action of antimycin A implicates cytochrome b as a component of the electron transfer chain in these particles. The fact that the DPNH-diaphorase reduction, a system that does not involve cytochrome b, is inhibited only 11 % (table IV), indicates that inhibition is specific to the cytochrome b site.

It is interesting that amytal, which inhibits electron transport between DPNH and the flavoprotein moiety in animal systems (2), was not effective in these particulates. Chance and Hackett (3) also noted the ineffectiveness of amytal in plant mitochondria.

SPECTRA OF APPLE PARTICLES: The suspensions of particles isolated from the alkaline homogenates

TABLE IV

INHIBITION OF ELECTRON TRANSFER BY CYTOPLASMIC PARTICLES*

INHIBITOR	DPNH OXIDASE**	DPNH-CYT. C** REDUCTASE	DIAPHORASE**	SUCCINIC-CYT. C** REDUCTASE
		*% Inhibition***		
Antimycin A***	76	77	11	78
Amytal†	4	2	2	...
		% Inhibition of succinoxidase††		
	10 MIN	1 HR	2 HR	
Antimycin A	66	40	21	

* The particles used for DPNH oxidase, DPNH cytochrome C reductase and diaphorase were from homogenates of pH 9.9. The particles used for the succinate assays were isolated from homogenates of pH 8.4.
** Assays were 3-minute spectrophotometric determinations as described in the text.
*** Antimycin A (0.5 μg) in 95 % ethanol was used in the cuvettes and Warburg vessels. An equivalent amount of 95 % ethanol was used in the blank.
† Amytal (3.6μ moles) dissolved in 95 % ethanol was used per cuvette. An equivalent amount of 95 % ethanol was used in the blank.
†† Assay was by Warburg manometric techniques. Assay system is described in text.

were very green and quite low in protein content (approx 2 mg/ml of suspension). It was therefore difficult to observe the spectra of the cytochromes in these particles. When the particles (derived from homogenates of pH 9.9) were concentrated from 3 kilograms of tissue, however, and the chlorophyll extracted by successive treatment with 25 volumes of cold (−15° C) acetone, until no green pigment was visible to the naked eye, it was possible to obtain difference spectra showing certain of the cytochrome bands.

The spectra shown in figure 2 are the difference spectra before and after adding dithionite to acetone-extracted concentrated particles. Absorption bands at 530, 560, and 605 mμ are clearly visible in these spectra. This corresponds to the β and α bands of cytochrome b and the α band of cytochrome a_3 (2). There is no obvious absorption band visible at 550 mμ to correspond to the α absorption peak of cytochrome c. Close scrutiny of this curve, however, reveals a hint of slight absorptions at 550 mμ and 520 mμ the α and β absorption peaks for cytochrome c. Since cytochrome c is the most soluble cytochrome it is possible that most of it has been extracted in the homogenization procedure. The residual cytochrome c bands may be masked by the much larger cytochrome b peaks. Skewing the cytochrome a_3 absorption band at 605 is due to the residual chlorophyll which tends to distort the band towards the higher wavelengths. In general, the curve is pitched sharply toward the higher wavelengths. This is due to the effect of dithionite on the overall absorption characteristics of the sample. For the most part, the absorption pattern observed in many plant cytoplasmic particles, demonstrating electron transport chain activity (7), was also seen in these apple particles.

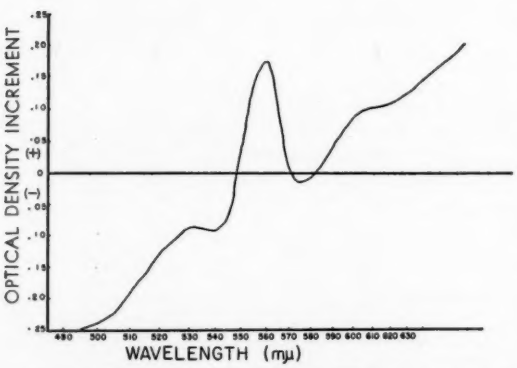

FIG. 2. Reduced minus oxidized difference spectra of apple particles obtained as outlined in text. The light path was 1.5 cm but was equivalent to an effective light path of about five centimeters resulting from a threefold intensification caused by endogenous scatter. The spectrophotometer used an end-window phototube and was specially designed to read through dense samples (1).

DISCUSSION

It seems that the dehydrogenases of the Krebs cycle, [as judged by succinate activity and oxidations of other Krebs cycle acids (data unpubl)], are better preserved by homogenates of pH 8.4, while the electron transport chain components are better retained in homogenates of pH 9.9. The characteristics of the pH 9.9 particles are similar to the ETP particles of Green and Crane (6) and therefore represent altered mitochondria. Since there is a basic difference in the localization and in the ease of extraction or solubilization of the Krebs cycle dehydrogenases and the electron transfer chain enzymes in the mitochondria, as noted by Green and Crane (6) and Lehninger (11), the action of alkali of pH 9.9 in these experiments, probably involves complete or partial removal by solubilization or otherwise of the substrate-level enzymes of the Krebs cycle. Even succinic dehydrogenase, a very insoluble system, is solubilized by a pH greater than 10 (6). The solubility effect may account for the lower nitrogen values of the particulate preparations from the more alkaline homogenates. On the other hand the much greater nitrogen content of the particulates from homogenates of low pH (6.0 and 6.9) is attributed to adsorptions of extraneous proteins that serve to inactivate the mitochondria. The greater activity of the electron transport chain in the pH 9.9 particles may be due to their more ready access to exogenous substrates in vitro, without interference of intervening layers of dehydrogenases which act as permeability barriers or regulatory controls.

Another way of looking at these data is simply to assume partial denaturation of the succinic dehydrogenase and other Krebs cycle substrate level enzymes, by the very alkaline homogenates. The more rugged electron transport enzymes encased as they are in phospholipid sheaths (11) are not injured and are in fact stimulated by the more alkaline buffers. One basis for this stimulation may be the purification of the enzymes since particles obtained from more alkaline homogenates have a lower specific nitrogen content. Another basis for stimulation may be related to an uncoupling effect caused by the high pH. Since the electron transport chain components are directly concerned with phosphorylation they may readily become uncoupled in very alkaline homogenates. As noted previously, oxidative phosphorylation was not observed with particles from pH 9.9 homogenates in the span from cytochrome c to oxygen, indicating that the system was uncoupled.

There is always the question whether or not the observed reductions of cytochrome c by DPNH and succinate are artificial extra-mitochondrial pathways. Inhibiting these reactions by antimycin A seems to support the view that the systems are intra-mitochondrial. Antimycin A inhibited both DPNH oxidase and DPNH-cytochrome c reductase about 80%, which indicates that both these oxidations proceeded through cytochrome b. The antimycin A effect on succinoxidase seemed less conventional since there was a loss of inhibition with time from a value of 66% to

21 %, after 2 hours. The length of time of the experiment may have somehow caused gradual degradation of antimycin A rendering it ineffective. However, it is also possible to explain the decline of inhibition by the gradual shunting of electrons around cytochrome b either by a secondary internal pathway or perhaps by means of some adsorbed microsomes. Shunting was not observed with the DPNH oxidase, DPNH-cytochrome c reductase, and succinic-cytochrome c reductase systems, because these assays were run for only 3 minutes.

Summary

A study of the isolation of cytoplasmic particles from apple homogenates of different pH (6.0–10.7), revealed that enzymatically active particles could be obtained only from alkaline homogenates. Particles isolated from homogenates of pH 9.9 gave the best electron transport chain activity while particles isolated from homogenates of pH 8.4 showed the best succinic-cytochrome c reductase and succinoxidase activity.

The cytochrome oxidase activity of these particles showed typical carbon monoxide inhibition and light reversibility. Antimycin A inhibited DPNH oxidase, DPNH-cytochrome c reductase and succinic-cytochrome c reductase, but did not inhibit diaphorase activity.

The difference spectra of the particles reduced with dithionite, after acetone extraction to remove chlorophyll, revealed absorption bands at 560, 530, and 605 mμ, which corresponds to the α and β bands of cytochrome b and the α band of cytochrome a_3. These data lead to the conclusion that the apple fruit has the usual type of electron transfer chain.

Acknowledgments

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ACTIVATION SPECTRA OF 700 mμ ABSORPTION CHANGE IN PHOTOSYNTHESIS

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In previous papers (1–3) we reported the occurrence of a light induced fast reversible absorption decrease around 700 mμ in photosynthetic organisms. The effect was ascribed to the photochemical bleaching of a pigment (P700) present in low concentration followed by a spontaneous regeneration in the dark. Such a mechanism predicts that the average concentration level of P700 is determined by the balance between photochemical destruction and dark restoration. However, we later observed with the blue green alga Anacystis (4) that light of certain spectral composition (red) raises rather than lowers this steady state level, and in this respect functions antagonistic to light of other wavelengths (far red). The following paper describes experiments in which we have attempted to determine activation spectra of the fast photochemical bleaching, as well as of the light induced increase of steady state absorption. Other observations concern the intensity relations between the two light effects, and the fraction of absorbed light possibly funneled through P700 turnover.

METHODS

Our difference spectrophotometer and its mode of operation were recently described (1). We modified the existing path of the actinic light to incorporate a monochromator. The mirror Mi [cf. fig 1 in (1)] was replaced by a small grating (Farrand 2″ × 3″). The entrance slit was mounted immediately behind the aperture in the two rotating discs. Two lenses served respectively to collimate the beam and to refocus it on the exit slit. Mounted close behind this slit, a lens imaged the grating onto the reaction vessel. A small Xenon arc (Hanovia 150 W) was used as an actinic light source, its discharge being focussed on the entrance slit. The half bandwidths of the monochromator, for the most frequently used slitwidths were 10 or 20 mμ. Over the wavelength area between 450 and 750 mμ the light output of the actinic illumination system was sufficiently constant to make corrections superfluous. The data shown in figures 2 to 5 are therefore simple plots of the recorder deflections. The described modification was about the best we could arrange with the already available design. Aside from biological limitations, such as settling or instability of the samples, the resolution and accuracy of the experiments were restricted by the rather limited spectral purity and (or) light intensity.

We may briefly recapitulate the two arrangements by which our difference spectrophotometer is used to observe light induced absorption changes (for more elaborate descriptions cf. 2, 4):

In the short dark time arrangement, the detecting device can be used to alternately observe the sample under study and an optical density wedge (adjusted to equal transmittancy in the absence of actinic illumination). At each switch-over moment from sample to reference beam (generally about 100 times/sec) the detector is briefly darkened and the sample hit by a flash of actinic light. Such a fast sequence of flashes is virtually equivalent to continuous illumination. Therefore, changes of absorption induced by admitting the actinic beam can be considered as steady state phenomena.

In the long dark time arrangement the detecting device observes the sample twice in each cycle: once immediately before the flash (observation B), and once immediately after the flash (observation A). The flashes occur at time intervals adjustable between 50 and 200 m.seconds depending upon the speed of the rotating discs. During the dark time (long enough to allow considerable processing of eventual photoproducts formed during the flash) the detecting device observes the reference beam in which the optical density wedge is placed (observation W). All observation periods last an equal time interval of about 0.1 of the cycle. Difference signals A-B indicate fast reversible or stepwise effects. Difference signal B-W relates the absorbancy of the sample at the end of each dark period (B) to the absorbancy level (W) prevailing before actinic light was added (to which the density of the wedge was equalized). The B-W signals therefore indicate changes of background absorption level and would a priori be expected to follow a similar pattern as the above short dark time observations.

An important point which should be emphasized here concerns the relative weakness of the actinic illumination in the present arrangement: in our first apparatus [described in (2)] which allowed only the measurement of fast reversible (A-B) absorption changes, a simple lens system of high aperture was used to provide the actinic illumination. Intensities close to those required for photosynthetic flash saturation could be obtained with the high pressure mercury arc employed. The more complex arrange-

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ment of the apparatus described in (4) necessitated the use of a less efficient optical system and incorporation of a monochromator further weakens the actinic illumination (in the long dark time arrangement we measured an integrated intensity of ~ 1000 ergs cm^{-2} sec^{-1} for a half band width of 20 mμ). The full detecting beam, which irradiates the sample continuously during the experiment was five-to tenfold stronger than this actinic light. In many instances therefore an interpretation of the effects brought about by the actinic flashes requires due consideration of the possible influence of the detecting beam upon the sample: when the detecting beam is of low intensity the spectra closely approximate light-minus-dark conditions. If the detecting beam is of relatively high intensity (comparable to the integrated actinic illumination) the spectra must be considered as light plus or minus more light.

Results

Figure 1 shows a difference spectrum (changes of absorption as a function of detecting wavelength) obtained with a suspension of whole Anacystis cells in the short dark time (steady state) arrangement and activating light of 680 to 710 mμ. The most pronounced absorption change occurs as a negative asymmetric shift around 700 mμ. Note that the data of figure 1 closely match our earlier published difference spectra (2) of fast effects (A-B differences) in the blue green alga Nostoc. This indicates that conversions of identical components are involved. As in other spectra discussed in this paper, the effects in the blue, especially with red activation, are not too clear cut and often comprise drifts or irreversible shifts. Therefore, we shall leave these undiscussed at present. (In this experiment the absorption decrease caused by actinic light around 480 mμ did not revert after its removal. For this reason we did not draw the curve through these points.)

Figure 2 shows an activation spectrum of the short dark time (steady state) 700 mμ absorption change obtained with a piece of thallus of the red alga Porphyra (difference spectra measured with these algae closely resemble those obtained with blue green algae (2)). Three intensities of the detecting beam were used in three series of measurements, in each series the activating wavelength was varied between 400 and 725 mμ. The half band width of the actinic beam was 20 mμ.

We first consider the data plotted as filled circles which were obtained with the full intensity of the detecting beam. In this case almost all wavelengths of actinic light induce an increase of absorption at 700 mμ. (Only the area around 700 mμ induces a slight negative effect).

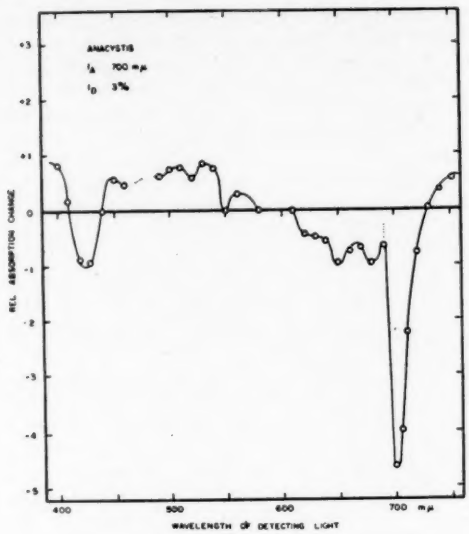
When the intensity of the detecting beam is decreased tenfold (dotted curve), the positive effects (induced by the same actinic intensities as used before) become smaller; their induction is confined to a wavelength area between 470 and 650 mμ. Shorter (400-470 mμ) as well as longer (650-710 mμ) wavelengths now induce a distinct decrease of absorption. A further tenfold decrease of Id causes all positive effects to disappear, accentuates the negative shift, and broadens the wavelength areas inducing it.

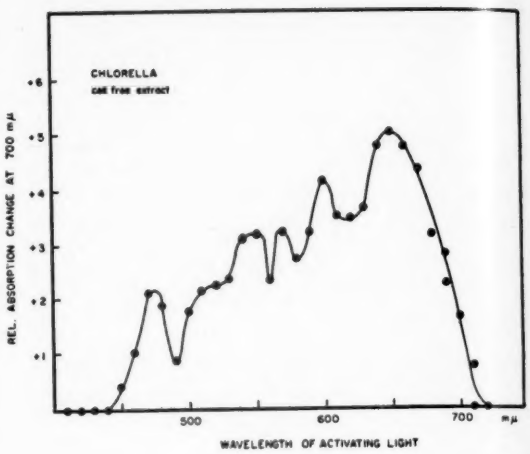
The pigments known to occur in Porphyra are chlorophyll a, carotenoids, and phycobilins (and possibly chlorophyll d). The maximum around 550 mμ in the activation spectrum with full detecting light indicates that phycoerythrin is the most important sensitizer of the positive shift. A shoulder at 630 mμ indicates sensitization by phycocyanin, whereas little effectiveness is observed in the blue where cholorophyll and carotenoids absorb. The red band of chlorophyll a does not appear to play a role in sensitizing the positive shift: wavelengths beyond 675 mμ cause a decrease of absorption. The negative shifts, predominant in weak detecting light, seem to be induced by chlorophyll and carotenoid, but the red activation maximum (close to 700 mμ) does not coincide with the chlorophyll a absorption maximum around 675 mμ. As will be discussed later in this paper, we tend to ascribe the position of this maximum mainly to interaction between positive and negative effects in this wavelength area.

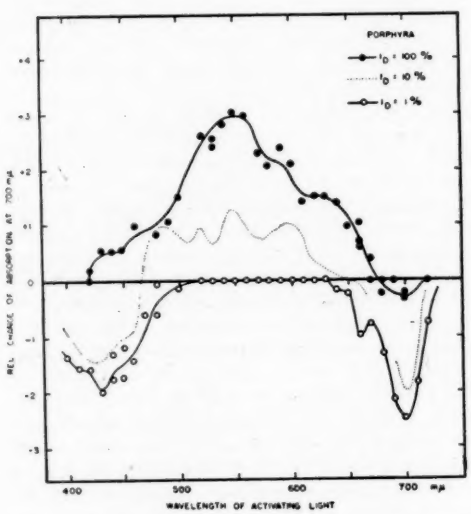
Figure 3 shows an activation spectrum of the positive shift as observed in the short dark time arrangement with a cell free extract of Chlorella (the supernatant obtained after grinding the algae with alumina and centrifugation). Effectiveness clearly is maximal at 650 mμ, the absorption maximum of the accessory pigment (in this case chlorophyll b). Although we do not ascribe much significance to details of the spectrum, a definite hump on the long wavelength side and an extension of effectiveness to (and possibly slightly beyond) 700 mμ appears significant. That is, it appears that in addition to chlorophyll b there is participation of part of the chlorophyll a in sensitizing the positive shift. We presently leave the short wavelength part of the activation spectrum undiscussed.

Figure 4 shows activation spectra of the 700 shift obtained with Anacystis and the short dark time arrangement.

The same organism was used in experiment (fig 5) which, in contrast to the previous experiments, was made with the long dark time (~75 m sec) experimental arrangement (cf. methods). The B-W signals which indicate changes of the background absorption level are plotted as circles in figure 5 and follow a similar pattern as the data of figure 4. With full detecting light, actinic wavelengths between 500 and 675 mμ, induce a strong increase of absorption at 700 mμ. Light of wavelengths below and above these limits induces small negative shifts. With weak detecting light, no positive absorption changes occur at all, but the negative effects are enhanced and the wavelength areas inducing these broadened. Analogous reasoning as used above in connection with the similar results of figures 2 and 3 leads to the conclusion that positive changes, if observed, are sensitized by accessory pigment (phycocyanin) whereas negative shifts are caused mainly by chlorophyll a and







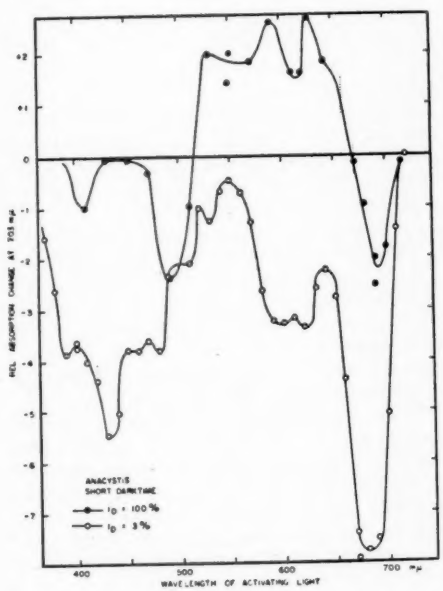


FIG. 1 (*top left*). Short darktime measurement of difference spectrum induced by far red light (680-720 mμ) in a sample of Anacystis. Detecting light intensity 3 % of maximum.

FIG. 2 (*bottom left*). Activation Spectra of the absorption change at 700 mμ at three intensities of the detecting beam I_D. Porphyra thallus. Short darktime arrangement.

FIG. 3 (*top right*). Activation spectrum of the positive absorption change at 700 mμ observed with a cell free preparation of Chlorella. Short darktime arrangement.

FIG. 4 (*bottom right*). Activation spectra of the absorption change at 700 mμ at two intensities of the detecting beam I_D. Anacystis whole cells. Short darktime arrangement.

carotenoid. Note, however, that in experiment (fig 4) there is also a significant sensitization of negative effect by phycocyanin.

In addition to the B-W signals we plotted in figure 5 (crosses) the simultaneously recorded A-B differences (fast effect). This fast effect appears to be induced mainly by the far red (650-700 mμ) and the blue part (400-500 mμ) of the spectrum and to have negative sign only. In contrast to the B-W differences (and the short dark time differences cf. figs 2-4), the intensity of the detecting beam (Id) influences the activation spectrum of the A-B effects only to the extent that with weaker Id the effects tend to increase (fig 6). For clarity we therefore plotted only one of the A-B activation spectra measured in experiment (fig 5).

Although the data are not too accurate, they definitely indicate that the fast reversible decrease of absorption at 700 mμ is mainly sensitized by chlorophyll a and possibly carotenoids. In many instances, however, especially in aged or poisoned algae (such as used in experiment fig 4) phycocyanin sensitizes the fast negative effect to a greater extent than shown in figure 5 (and then induces with low I_D also a negative short dark time or B-W signal). A final remark concerns the incongruency around 660 mμ between the A-B and B-W activation spectra in figure 5. Compared to the dotted curve which tends to follow the red absorption band of chlorophyll a this peak is shifted toward the red in the full curves. We ascribe this shift of the B-W effectiveness maximum to the overlapping of positive (phycocyanin) and negative (chlorophyll) activation (No such interaction is involved in the A-B activation spectrum). This same incongruency readily explains why in our earlier data [(1), fig 7] obtained with wide actinic wavelength bands, the sign of the two types of change could be either the same or opposite.

Intensity Relations: The fact that often the measured curves do not quite match the absorption spectra of the pigments likely to be involved could be caused by several factors besides the limited accuracy of the experiments. First, in order to obtain measurable deflections, rather dense samples had to be used. The two beams enter the sample at a 90° angle and each may traverse a layer of algae before hitting cells which are also traversed by the other beam. Such a screening effect might lead to a relative decrease of effectiveness in regions of strong absorption. [As was shown earlier (2); the difference spectrum will also be distorted for this reason].

Second, data like those of figures 2 to 5 can of course only be interpreted if the intensity dependence of the involved effects is known. The chlorophyll sensitized negative changes depend mainly upon intensity Ia, but strong detecting light diminishes the effect of the actinic beam. An example of this is shown in experiment (fig 6) made with the short darktime arrangement. Here the effects brought about by the actinic beam were considerably decreased in the strongest available detecting light.

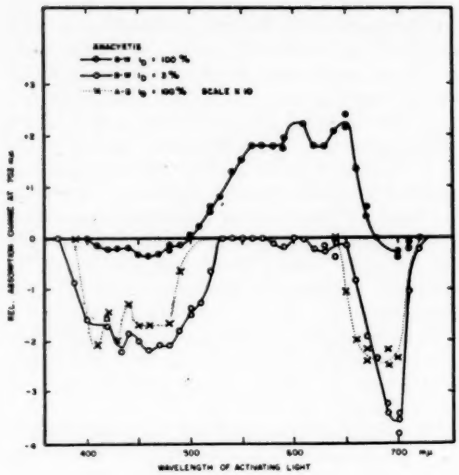
Such interaction between the two beams of closely identical wavelength should be expected if one accepts the existence of a pigment with an absorption band at 700 mμ which is temporarily bleached as soon as it receives light (either by direct absorption or via transfer from vastly more abundant chlorophyll a): There is no reason to assume a difference between the actions of the flashing actinic light and the continuous 700 mμ detecting light. The latter therefore will cause a continuous breakdown of P700, which will be counteracted by the dark backreaction. The more P700 is bleached, the less material remains available for photochemical conversion and the faster the backreaction will go. Therefore, at any intensity of Id an equilibrium between photochemical breakdown and dark build up will be attained. With strong Id, there will be a low steady state concentration of P700. At the moment the actinic light is added far red light will find little material to bleach and the negative effect will be small.

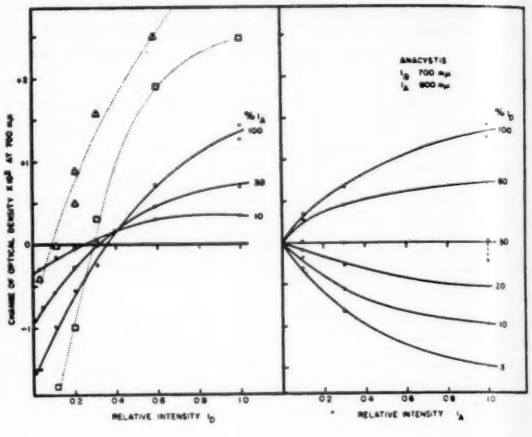
The positive effect on the other hand may be conceived as an accelerated restoration of P700 induced by phycocyanin absorbed light. Then a detecting beam strong enough to keep a sizeable fraction of P700 in the bleached state will provide abundant substrate for this restoration process. This readily explains the observation in figures 2, 4, 5, and (1) figure 8 that increases of absorption occur only if strong enough detecting light is used, and become larger the higher intensity Id.

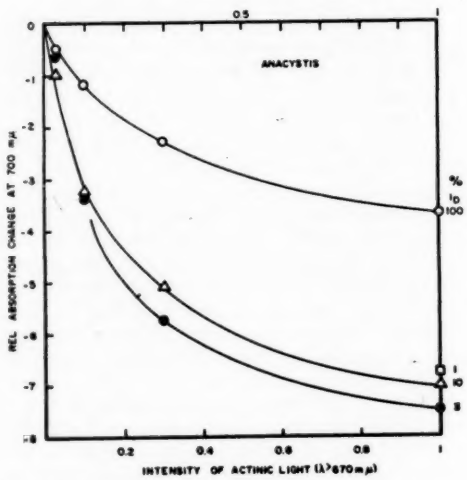
However, in hardly any sample of Anacystis that we tried did light of wavelengths mainly absorbed by phycocyanin induce positive changes exclusively. To obtain a reasonable range in our study of intensity relations we used a rather broad spectral area for the actinic illumination (580-620 mμ). Not only is part of this light directly absorbed by chlorophyll a but the variability of the phenomena described below and in figures 4 and 5 indicates that dependent upon algae and conditions a smaller or larger fraction of the phycocyanin can also contribute to the negative effect (Possibly by transfer of its excitation to chlorophyll a).

On the basis of these assumptions we can qualitatively explain the intensity relations observed in experiment (fig 7): three intensities of actinic light were measured each with six intensities of the detecting beam. The two plots of full lines depict the results: positive absorption changes were only observed if the detecting beam was stronger than one-third its maximal intensity. The changes increased with increasing Ia as well as Id, but approached a maximum. With lower intensities of Id the changes became increasingly negative and again more the stronger Ia. Most striking is that at Id 30 % the actinic beam induced no effect at all, regardless of its intensity.

This peculiar interplay between the two light beams of different color can only be explained by assuming that two antagonistic light reactions and at least one dark step (such as the spontaneous restoration) determine the concentration of P700. A more formal description of this interplay has been given in







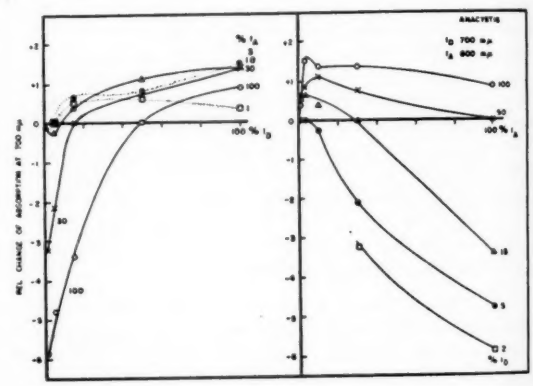


FIG. 5 (*top left*). Activation spectra of absorption changes at 700 mμ measured with the long darktime arrangement and whole Anacystis cells. Dots: B-W Signal I_D 100 %, Open circles: B-W signal I_D 3 %. Crosses: A-B Signal I_D 100 %.

FIG. 6 (*bottom left*). Dependency of the magnitude of the absorption change at 700 mμ upon the intensities of detecting (I_D, 695-705 mμ) and activating (I_A, 680-700 mμ) lights. Short dark time arrangement.

FIG. 7 (*top right*). Full lines: Dependency of sign and magnitude of the absorption change at 700 mμ upon the intensities of the detecting beam (I_D, 695-705 mμ) and the actinic light (I_A, 580-620 mμ). Dotted lines: Two runs (I_A 580-620 mμ full intensity, I_D varied) made with samples from the same culture on two consecutive days. The third day no positive effects were found with this culture. Short darktime arrangement.

FIG. 8 (*bottom right*). Experiment similar to that of figure 7 performed with a suboptimal sample of Anacystis.

(5). A quantitative study of these phenomena is restricted by the variability of the biological material: It seems that the strongest positive effects are found in healthy young algae. With age or deterioration of the culture positive effects require a higher Id to occur or the phenomena become quite complex: The two dotted lines in figure 7 (left) were obtained with algae from a culture tested on two consecutive days (Ia 100 %). The first day (triangles) only slight negative effects and large positive ones occurred; the point of zero change was at Id=10 %. The second day (squares) the negative effects were larger and the smaller positive effects tended to saturate at higher intensity of Id; the zero point was shifted to Id= 30 %. The next day, all positive effects had disappeared and only negative shifts were observed. Figure 8 illustrates the complexity of the interrelations encountered in several instances which we tend to correlate with the use of suboptimal algae (although no simultaneous measurements of photosynthetic rate were made). In this experiment at certain levels of detecting illumination, positive shifts occur only over a range of actinic intensity, lower as well as higher values leading to negative deflections. We should mention again that such negative changes, sensitized by accessory pigment show a similar kinetic behavior as those sensitized by chlorophyll (i.e., absence of transitory overshoots and occurrence of an A-B long darktime signal).

It is obvious that in cases where intensity relations like those of figure 8 prevail, the significance of activation spectra will be quite ambiguous. On the other hand, there is good agreement between intensity relations such as depicted in figures 6 and 7 and the activation spectra observed in experiments (figs 2 & 4).

Discussion

The long darktime experiments indicate that the bleaching of P 700 is a direct photochemical act (we could confirm this in preliminary direct oscilloscope observations). In contrast, no fast positive effect has so far been observed in the red part of the spectrum.[3] Even in cases where large increases of absorption are indicated by the B-W signal, the A-B differences are negative or zero. (This is likely if phycocyanin transfers some of its energy to chlorophyll.) This means that, in the process sensitized by accessory pigment, there seems to be a delay between illumination and the formation of P700. This delay, of at least several milli seconds indicates the mediation of dark reactions. Apparently, the photochemical act initiating the 700 mμ absorption increase is not itself characterized by a pigment conversion at some other wavelengths observable with our apparatus. Although our experiments yield only indirect evidence on this, we consider it quite likely that the photochemically induced reformation of P700 (positive effect) involves an entirely different mechanism than the spontaneous (dark) backreaction. The negative shift and spontaneous return can be observed even in photosynthetically completely inactive preparations and nearly always appears as a simple reversible (A-B, B-W or short darktime) recorder deflection. The positive effect is only found in healthy algae or fresh preparations and usually shows transitory phenomena which indicate the interplay of several reactions [cf. (1) figs 7 & 8].

An attempt to clarify the course of events observed with our long darktime arrangement is shown in figure 9. It is assumed that the quantity studied is the relative amount of the absorbing form of P700. We accepted that in darkness all of the pigment is in the absorbing state, the bleached fraction becoming larger the higher the intensity of the detecting beam. (This decrease of P700 caused by the detecting beam alone cannot be observed with our present apparatus.) Only in strong detecting light is enough bleached

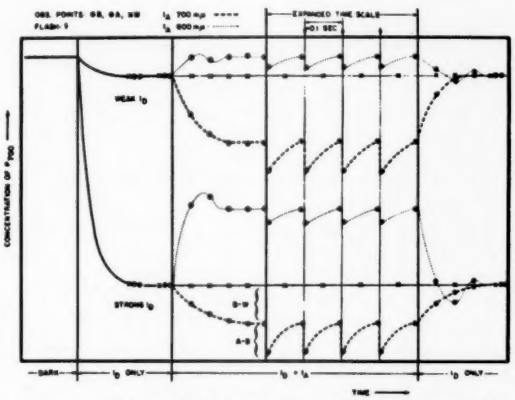


Fig. 9. Schematic diagram illustrating the probable time course of the concentration of P700 in long darktime experiments with Anacystis. Signal W (i.e., the reference beam, indicated as crosses) is adjusted to equal signals A (observed after each flash, open circles) and B (observed before each flash, dots) in detecting light only. The time course followed with red actinic light is indicated as a dotted curve, that with far red actinic light as a dashed one. Four individual flash dark cycles are drawn on an expanded time scale to illustrate the computation of A-B signals in addition to the B-W differences.

[3] This does not necessarily mean that no positive A-B signals can be observed. The discussed experiments were performed with a certain rotation speed of the discs. By lowering this speed, all events are congruently stretched out in time: flashtime, darktime, observation periods, as well as the delay between flash and observation A. Under such conditions the fast decrease may escape measurement and the photoinduced reformation of P700 yield a positive A-B signal. It must be clear then that the information yielded by our present apparatus is limited to yielding crude cross sections through probably complex time courses.

material present to allow a measurable shift of the system by adding red [600 mμ light (leading to a positive B-W signal)]. The new equilibrium is attained via transitory oscillations. Flashes of far red light temporarily lower the amount of unbleached pigment (which yields an A-B signal). The concentration of P700 will remain decreased when the darktime between flashes is too short to allow the backreaction to run to completion (which then yields a negative B-W signal).

Figure 9 should clarify that in steady state flashing light, as soon as the B-W signal becomes constant, a deflection of the A-B recorder indicates a repetitive phenomenon: the amount of pigment bleached and again restored in each individual light-dark cycle. If the flashes are given at a rate of n per second, the total amount of conversion corresponds to n times the A-B signal. For instance, in experiment (fig 6) the largest A-B shift was about 3×10^{-4} O.D. units and at a flash rate of 15 × per second, some 5×10^{-3} O.D. units of pigment turned over per second. If one accepts P700 to have a molar extinction coefficient of 10^5 (like the red band of Chlorophyll a), one can express this rate in moles per second. When the integrated intensity of Ia (assumedly totally absorbed), is expressed in Einsteins per second, the ratio between the two values indicates the quantum yield of the conversion. Quantum requirements computed in this way ranged between one and five.

The complex and different geometry of the two light beams, the rather long duration of the observation periods (10 m sec) relative to total dark period (~80 m sec) in our present set up, as well as screening and self absorption effects, make such computations quite hazardous. But the order of magnitude of the numbers indicates that we probably are dealing with a gross process rather than with a small loss side effect (such as fluorescence or luminescence are): if, in photosynthesis, all light absorbed by chlorophyll a were funneled through P700 conversion, a quantum yield of unity would be expected. Our computed numbers actually are maximum values, since the actinic beam not only induces the reversible changes, but in addition maintains the background level of P700 (B-W signal). Not knowing the mechanism or time constants of the back reaction, we cannot guess to what magnitude this correction might amount. For this same reason, the rate of pigment turnover represented by the positive B-W signals cannot be estimated directly. However, it is reasonable to assume a turnover rate of similar magnitude for the conversions sensitized via phycocyanin. We therefore are tempted to assume that both effects have functional significance in the process of photosynthesis.

Summary

Activation spectra were measured of the light induced absorption changes at 700 mμ in photosynthetic organisms. The direct photochemical bleaching of the involved pigment appeared to be sensitized mainly by chlorophyll a and possibly carotenoids. The opposite effect, accelerated restoration of this pigment, was sensitized mainly via accessory pigment (such as Phycocyanin in Anacystis) and mediated by dark reaction steps. The data suggest that both light reactions are functionally involved in photosynthesis.

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PRODUCTION OF AUXOTROPHS IN A DUCKWEED, SPIRODELA POLYRHIZA[1,2]

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Although auxotrophic mutant strains of microorganisms have been used extensively for growth factor and metabolism studies, similar use of biochemically deficient mutants of multicellular plants has been limited. Phinney (17) has employed genetically dwarfed mutants of maize for detecting gibberellic acid. Artificially induced mutants of higher plants, in which the genetic lesions are expressed as nutritional deficiencies, have been studied to an even lesser extent. Langridge (10), by irradiation of seeds of a crucifer, *Arabidopsis thaliana* (L.) Heynh, obtained several X_2 generation lethals, some of which appeared to be auxotrophic mutants since they responded to chemical supplements. Langridge suggests the use of such biochemical mutants for chemical and genetic investigations. References to the biochemical effects of ionizing radiation on plants are included in a recently published bibliography (18).

The objective of this investigation was to develop new information on plant metabolism through study of nutritionally dependent strains of the duckweed *Spirodela polyrhiza* (L.). Methods for producing auxotrophic forms of *Spirodela polyrhiza* by X irradiation and for selecting these from a mixed culture are described. Since *Spirodela polyrhiza* is a diploid organism which reproduces vegetatively, detection of recessive mutations is not likely. However, the less common dominant mutations and certain chromosomal aberrations such as translocations, inversions and interstitial deletions would be recognizable and should persist through vegetative reproduction. Landolt (9) has shown that abnormalities can be produced in duckweed cultures which persist through several vegetative generations. In this work, we have agreed to call any nutritionally dependent strain which persists more than 50 vegetative generations an auxotroph.

Before artificially induced auxotrophs could be detected, it was necessary to determine conditions for optimum growth of the prototroph. In addition, the growth requirements of one of the auxotrophs isolated during this work were determined and are discussed.

MATERIALS AND METHODS

MEDIA: Four growth media for *Spirodela polyrhiza* are referred to by letter designation in this report. They are as follows: Medium A (table I) was a mineral salts solution adapted from one described by Nickell and Finley (16). Iron was supplied as a chelate of ethylenediaminetetraacetic acid, Geigy's "Sequestrene" NaFe (13 %). Most supplements to this and subsequent media were made with "CfP" quality chemicals obtained from California Corp. for Biochemical Research. Medium B was Medium A plus glucose, 3 g/liter; Medium C was Medium B plus cystine, 5 mg/liter; choline chloride, 10 mg/liter; and gibberellic acid, 1 mg/liter. This was the basal medium used in auxotroph selection. Medium D was Medium C plus "Difco" certified Bacto yeast extract, 0.5 g/liter. This was the supplemented medium used in auxotroph selection.

CULTURE: A strain each of *Spirodela polyrhiza* and *Lemna minor* (L.), were isolated from a mixed duckweed culture purchased from Carolina Biological Supply Co. Pure cultures were produced from single fronds by brief immersion in 70 % ethanol followed by immersion for 5 minutes in 1.0 % calcium hypochlorite solution. The fronds were then washed

TABLE I

MINERAL SALTS MEDIUM (MEDIUM A)

SUBSTANCE	CONC/L*	
$Ca(NO_3)_2 \cdot 4H_2O$	1.310	g
$MgSO_4 \cdot 7H_2O$	544	mg
KH_2PO_4	155.2	mg
KNO_3	553.0	mg
NH_4Cl	1.5	mg
H_3BO_3	3.17	mg
$CuSO_4$	0.0885	mg
$MnSO_4 \cdot H_2O$	1.703	mg
$ZnSO_4 \cdot 7H_2O$	0.433	mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0975	mg
Sequestrene NaFe (13 %)	38.75	mg

* These concentrations were obtained by dilution from 400-fold concentrated stock solutions. The pH was adjusted to 5.5 with potassium hydroxide.

[1] Received March 18, 1960.

[2] Contribution No. 602 from the Central Research Department, Experimental Station, E. I. du Pont de Nemours and Co., Wilmington, Del.

several times with sterile water and placed on medium A.

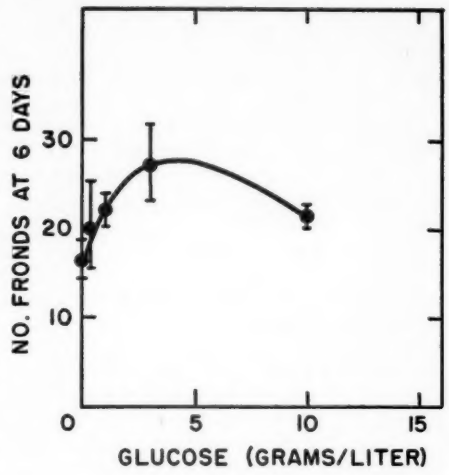
Three-frond clusters were subcultured at intervals of 10 days for a period of about one year prior to use in this research. Cultures were grown at 30 ± 1° C under either 400 or 2,000 ft-c of nearly continuous lighting (23.75 hr day length). As the growth rate at 2,000 ft-c was slightly faster than at 400 ft-c, the higher light intensity was adopted for all experiments described here. In preliminary experiments, growth under entirely fluorescent light was compared to growth under fluorescent plus incandescent light. As no differences in frond size, multiplication rate, or morphology were observed, entirely fluorescent lighting was chosen for the bulk of this investigation.

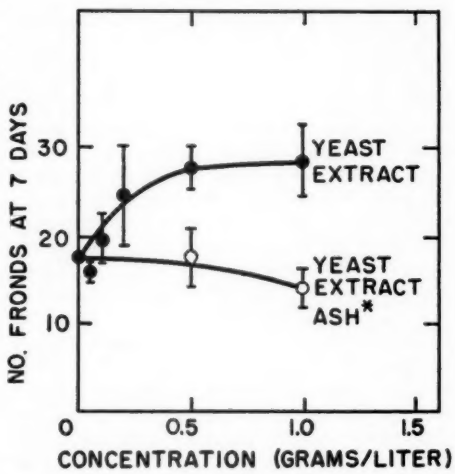
The growth experiments were conducted in 50 ml Erlenmeyer flasks that had been charged with 17 ml of medium, stoppered with gauze-covered cotton plugs and autoclaved at 120° C for 20 minutes. An experiment showed that growth on autoclaved glucose-containing medium was equivalent to growth on autoclaved medium plus filter-sterilized glucose. Inocula were prepared by selecting young fronds each possessing a small bud, rinsing with sterile water, and transferring a single frond with bud to a flask of sterile medium. For each experimental condition five or six replicate inocula were used.

Growth Measurements: In all experiments, growth was measured by counting the number of fronds and buds present at the end of the growth period, usually 7 or 8 days. This fairly long growing period minimized possible errors which might arise from selecting inocula during different phases of growth. In some instances growth rates were obtained by measuring the slopes of log frond number vs. time.

Occasionally additional measures of growth were used to substantiate unusual effects. These included frond area of fresh plants and weight of dried plants. For measurements of frond area, a simplification of Gorham's method (5) was employed. Fresh plants were placed, roots up, on a bacteriological slide and blotted gently with a paper towel. The slide was interposed on a mask between a GE 1493 lamp and a photocell which were mounted at opposite ends of an opaque cylinder. Light not absorbed by the duckweed passed through the slide and impinged upon the photocell. The photocell current, which was read on a galvanometer, was inversely proportional to the total frond area.

X Irradiation: A 2 mev, 500 w Van de Graaff accelerator fitted with a water-cooled gold target was employed for generating X rays. Dosimetry measurement (8) by irradiation of a flask of ferrous sulfate solution at 50 inches from the target indicated that 333 rads per minute were adsorbed. Erlenmeyer flasks containing 40 to 50 three-frond clusters of duckweed suspended on sterile water were irradiated for periods of 3 to 24 minutes, affording radiation doses of 1,000 to 8,000 rads per flask.





* VALUES REPRESENT THE AMOUNT OF YEAST EXTRACT ASHED.

Fig. 1 (*top*). Growth of Spirodela polyrhiza in Medium A. Effect of glucose concentration.

Fig. 2 (*bottom*). Growth of Spirodela polyrhiza in Medium B. Effect of yeast extract and ash of yeast extract.

Experimental Results

Effect of Sugars on Prototrophs: Cultures of *Spirodela polyrhiza* grew more rapidly and appeared healthier when a sugar was added to the mineral salts medium A. This effect was less pronounced at 2,000 ft-c than at 400 ft-c, which implies that the optimum light intensity for photosynthesis in this duckweed is greater than 2,000 ft-c. The effect of glucose concentration on growth rate is shown in figure 1. The

concentration of glucose for optimum growth appeared to be about three grams per liter, therefore this was chosen as basis for medium B. Cultures maintained on medium B had larger, greener fronds and much longer roots than cultures maintained on medium A. Sucrose at 10 g/liter in medium A also increased frond number, but the average frond area was only 8.3 mm² as compared with 17.7 mm² on glucose (medium B). Galactose stimulated growth rate slightly, and xylose inhibited.

Effects of Other Supplements: Water extracts of certain natural materials including sphagnum peat and horse and cow dung have been reported to stimulate growth of duckweed (5). Yeast extract and casein hydrolysate both were found stimulatory for growth of *Lemna minor* (L.), (6). Yeast extract was added to medium B to determine if factors other than sugar and those supplied by the mineral salts medium would promote growth of the wild-type *Spirodela polyrhiza*. It is evident from figure 2 that yeast extract stimulates growth best at about one gram per liter, based on the total frond number. Cultures grown on yeast extract at 1 g/liter, however, appeared abnormal, the fronds being extremely cupped, convex above and concave below. Those grown on yeast extract at 0.5 g/liter appeared of relatively normal shape, but larger and deeper green than those grown on medium A. Coconut milk and aqueous extracts of *Spirodela polyrhiza* were inhibitory at concentrations of 1 % and higher and were ineffective at lower concentrations. Ash of yeast extract was inhibitory, indicating that the stimulation probably was not due to a metal ion.

In efforts to duplicate the stimulation by yeast extract, a number of known metabolites and growth factors were tested as filter-sterilized supplements to medium B at what were expected to be appropriate concentrations. The list included amino acids, vitamins, purines, plant growth hormones, and kinetin. Most of these, including indoleacetic acid and kinetin, were without effect. Asparagine, which stimulated increase in frond number for Gorham's strain of *Spirodela polyrhiza* (5), was inhibitory for this strain. Methionine and 2,3-dimercaptopropanol were also inhibitory. Slight stimulations were observed with adenine sulfate and with *p*-aminobenzoic acid each at 1 ppm. Choline chloride, 10 ppm; gibberellic acid, 1 ppm, and cystine, 5 ppm, were definitely stimulatory, and their effects were somewhat additive (table II). Thus, medium C was formulated by adding these three compounds to medium B prior to autoclaving. Autoclaving did not diminish stimulation by the supplements.

The data in table II show that medium C produced stimulation greater than that produced by adding yeast extract to medium B. Adding yeast extract to medium C did not produce significant additional stimulation (medium D). In addition to increased frond number, stimulation by these materials is reflected also by increased total frond area. Root growth on the medium containing cystine was especially good.

Though there may be materials in yeast extract stimulatory for the growth of wild-type *Spirodela polyrhiza*, their effects probably would be completely masked by the presence of choline, cystine, and the possible presence of gibberellic acid. Accordingly, for selection of X ray induced auxotrophs of this duckweed, medium C was chosen as the minimal medium. The prototroph grows optimally on this medium but auxotrophs for factors not supplied by this medium grow poorly or not at all. Medium D, containing 0.5 g/liter yeast extract to supply possible growth factors not present in medium C, was chosen as the so-called completely supplemented medium. Auxotrophs for factors present in yeast extract can be maintained on this medium; naturally, medium D would not be complete for any auxotrophic strains requiring some factor not supplied by yeast extract.

Production and Selection of Auxotrophs: Three days after irradiation, fronds in flasks that had received 1,000 and 2,000 rads appeared to be growing and reproducing slightly faster on medium A than were the unirradiated controls. Those receiving 4,000 rads were growing slightly slower, and growth of those receiving 8,000 rads was considerably inhibited compared to growth of the controls. All samples at this time appeared morphologically normal. Ten days after irradiation all the duckweeds that received 8,000 rads of X rays were necrotic or dead; those that received 4,000 rads contained a variety of weird forms but most appeared to be surviving. The dose-response curve in figure 3 shows the number of surviving fronds and buds at 6 days post-irradiation. It was estimated that about 6,000 rads are necessary to kill about 90 % of the new buds emerging from the clefts of the irradiated fronds.

Viable buds emerging in cultures irradiated with 6,000 rads of X rays were separated and grown in individual flasks on medium D until 10 to 12 three-frond clusters were produced. Each cluster then was transferred to medium C. From those cultures on medium C which multiplied only slightly or produced

Table II

Growth of Spirodela Polyrhiza; Stimulation by Various Substances

Medium	Additions (mg/l)	Mean* frond No., 7 days	Area/frond mm²
A (mineral salts)	...	15.4	11.9
B (A + glucose, 3 g/l)	...	22.0	14.3
B + Yeast extract	500	27.2	17.6
B + Choline chloride (CC)	10	28.2	...
B + Cystine (Cys)	5	28.6	15.1
B + Gibberellic Acid (GA)	1	29.8	15.1
C (B + CC + Cys + GA)	10, 5, 1	36.4	...
D (C + Yeast extract)	500	38.6	...

* The mean number of fronds from six replicate two-frond inocula.

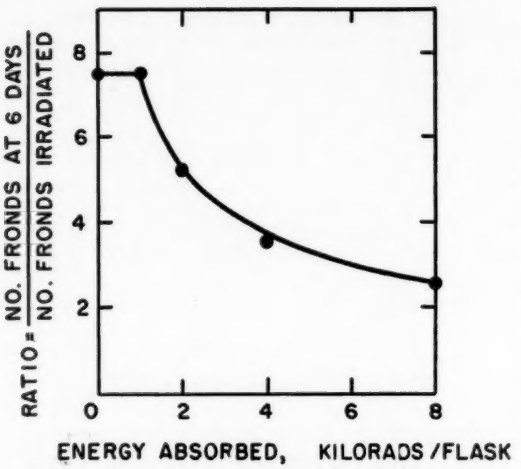


FIG. 3. X irradiation of *Spirodela polyrhiza*. Dose-response curve.

only very sickly fronds, a three-frond cluster was transferred to fresh medium D. Cultures which, after several additional transfers, consistently grew better on medium D than on medium C were considered to be possible auxotrophs. Five such varieties were obtained. The data in table III compare the growth of these varieties to that of the prototroph on four different culture media. The values represent the average number of fronds in five replicates.

Four of these strains were maintained through at least 10 transfers over a period of 3 months. Strain 301 was maintained through at least 75 transfers over more than 2½ years.

The growth characteristics of the natural strain of *Spirodela polyrhiza* and of strain 301 are shown in figure 4. The log growth for the natural strain is linear in time on all media (A–D), whereas log growth of 301 becomes nearly linear in time only on Medium D. Because the curves for strain 301 are not linear, frond doubling time, t_2, is calculated from the latter portion of the curves. Growth rates for the natural strain and strain 301 do not differ greatly on medium D. On this medium, t_2 for the natural strain is 1.50 days, as compared to $t_2 = 1.75$ days for strain 301. Comparison of t_2 values for these strains on other media reveals marked differences.

REQUIREMENTS OF STRAIN 301: This auxotrophic strain was chosen for detailed study. To gain an idea of the qualitative nature of the stimulatory factors, yeast extract was dialyzed in a cellophane bag against deionized water at 4° C for 20 hours. The material that passed through the membrane was stimulatory when added to medium C, whereas the undialyzable portion was somewhat inhibitory. The activity of the dialyzed material was not destroyed by boiling in water for 30 hours. Subsequent to this experiment it was observed that boiling the original yeast extract with 1 N HCl for 2 hours increased its stimulatory action.

The stimulatory factor in yeast extract was extractable from aqueous solution at pH 2, 7, and 10 by 1-butanol, but not by less polar solvents. Partial purification of the stimulatory factor was accomplished by a butanol extraction of acid-hydrolyzed yeast extract at pH 7. Evaporation of the butanol extract to dryness, redissolution, and filtration through Celite yielded a clear, light-yellow aqueous solution which was then lyophilized. The lyophilized powder stimulated the growth of the auxotroph as well at 100 mg/liter in medium C as did yeast extract at 500 mg/liter.

The above observations, together with the fact that stimulation was also obtained with both malt extract and hydrolyzed casein in medium C, suggested that strain 301 might be auxotrophic with respect to amino acids. Therefore, an amino acid supplement was prepared of the type used in culture of *Tetrahymena geleii* (13). This contained glycine (0.20 mg/ml), L-alanine (1.1 mg/ml), L-arginine · HCl (4.98 mg/ml, L-histidine · HCl · H_2O (2.38 mg/ml), L-isoleucine (2.75 mg/ml), L-leucine (6.9 mg/ml), L-lysine · HCl (6.75 mg/ml), L-methionine (2.50 mg/ml), L-phenylalanine (2.0 mg/ml), L-proline (5.00 mg/ml), L-serine (3.95 mg/ml), L-threonine (3.25 mg/ml), L-tryptophan (1.45 mg/ml), L-valine (1.63 mg/ml), and L-aspartic acid (2.45 mg/ml). This stock solution when added to medium C at 10 ml/liter stimulated growth better than yeast extract or acid-hydrolyzed casein. Little or no stimulation was observed when each amino acid was tested separately. Histidine was extremely inhibitory. When both the amino acid supplement and yeast extract were present in medium D, growth stimulation was even greater than that produced by either supplement alone.

In view of this apparently complicated situation

TABLE III

GROWTH OF AUXOTROPHS COMPARED TO PROTOTROPHIC SPIRODELA POLYRHIZA

STRAIN	MEAN FROND NO.*			
	MEDIUM A	MEDIUM B	MEDIUM C	MEDIUM D
Prototroph	21.6	30.8**	51.0**	53.5
262	4.2	8.6†	31.8**	41.4**
287	6.8	14.6***	33.8**	43.8
301	6.4	13.2**	21.8**	39.4**
338	20.6	28.8†	32.2	43.2**
349	19.2	40.6**	52.0†	62.4

* Mean number of fronds from each of 5 replicate two-frond inocula after 8 days.
** Significant for P = 0.01.
*** Significant for P = 0.02.
† Significant for P = 0.05.

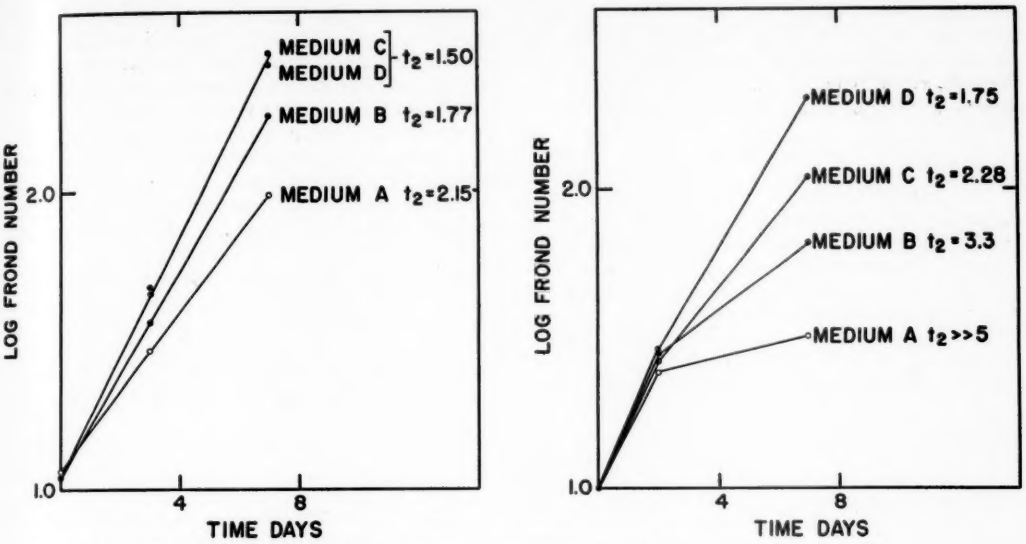


FIG. 4. Growth of *Spirodela polyrhiza*. a (*left*). Wild type. b (*right*). Strain 301.

with respect to stimulation by amino acids, it was decided to isolate the active materials from yeast extract. Countercurrent distribution of 2 g of the lyophilized butanol extract between butanol and water was effected on a 50-tube Craig countercurrent distribution apparatus. After 50 transfers, the fractions were collected in pairs, evaporated to dryness, and tested for biological activity. Two major peaks of high activity were found. The first, in tubes 9 to 14, represented a large amount of the lyophilized material; the second peak, in tubes 35 and 36, represented only a minor amount of the original material.

Material in the earlier peak gave optimum stimulation of the growth of strain 301 at 20 mg/liter, representing a fivefold increase in activity over that of the lyophilized powder. Paper chromatography of combined fractions 9 to 14 in butanol saturated with water (3) yielded a single ultraviolet-absorbing component at R_f 0.39 which corresponded closely to pure uracil. Spraying with ninhydrin reagent revealed three additional purple spots.

Microbiological assay (13) of fractions 13 to 14 with *Tetrahymena pyriformis* (Ehrenberg) strain W indicated that about five to ten per cent of the material was either uracil or uridine. Assay of pure uracil and pure uridine on strain 301 showed them not to be stimulatory factors.

Chromatographic separation of 150 mg of the material recovered from tubes 13 and 14 on a 20 × 28 cm sheet of Whatman 3 MM paper with butanol-acetic acid-water (23) yielded enough material in each of the three ninhydrin-positive areas to be tested biologically. Materials from two of these areas were inactive on growth of strain 301. A substance eluted from the third area promoted growth strongly at 10 mg/liter in medium C. Its value of R_f = 0.65 corresponded closely to leucine, isoleucine, or phenylalanine. By means of bacterial mutants obtained by the procedure of Adelberg and Myers (1) blocked in the synthesis of these respective amino acids, combined fractions 9 to 14 were shown to contain about 50 % L-leucine and 20 % L-isoleucine, but essentially no phenylalanine. Retesting of authentic samples of L-leucine and L-isoleucine in medium C individually showed them each to have a slight stimulatory effect on the growth of strain 301 though the stimulation by L-isoleucine could not be shown to be statistically significant to P = 0.05. However, when these two amino acids were mixed at 2 to 4 mg/liter each, they fully duplicated stimulation exhibited by fractions 9 to 14. When tested singly at higher concentrations (10 or 16 ppm) these two amino acids inhibited growth completely but exhibited no net effect when both were present in the culture medium.

The material in the second peak was found to be ether-soluble. Because of this, its possible identity as a sterol was considered. The combined contents of tubes 35 and 36 were evaporated to dryness, taken up in ethanol, and treated with an ethanolic solution of digitonin (4). The digitonide precipitate was collected by centrifugation, washed with cold alcohol, then dissolved in pyridine. Digitonin was precipitated by adding cold ether and removed by centrifuging. The supernatant was evaporated to dryness, and the solid residue when tested on strain 301 at 1 mg/liter in medium C fully duplicated stimulation of the material found in the second peak of the countercurrent distribution. Additional tests revealed that this material stimulates wild-type *Spirodela polyrhiza* also. Subsequent testing of pure steroids showed ergosterol, β-sitosterol, stigmasterol, and desoxycholic acid at 0.5 mg/liter all stimulate growth of *Spirodela*

polyrhiza. Both stigmasterol and desoxycholic acid were more stimulatory than the materials from the second peak of the countercurrent distribution; desoxycholic acid much more so. None of these steroids showed any effect on culture of *Lemna minor*.

Discussion

Culture of Wild-type Spirodela: The duckweed, *Spirodela polyrhiza*, was chosen for these studies because this miniature aquatic plant is easily cultured on sterile liquid media and the growth rate is rapid. Under the conditions employed here, the frond number doubles in less than 2 days.

To select auxotrophs by comparing their growth with that of the prototroph on various media, it was necessary to establish the best possible defined medium for culture of the prototroph. This work led to the formulation of medium C, which contained mineral salts, glucose, cystine, choline chloride, and gibberellic acid. Adding yeast extract to this medium (to give medium D) resulted in no significant further stimulation of the prototroph (tables II and III). Possibly the growth media might have been improved further by adding some ammonium salt, since discussion with Dr. W. S. Hillman has revealed that ammonium salts are a better source of nitrogen at pH 5.5 for certain Lemna species than are nitrates. However, Gorham (6) finds nitrate to be a satisfactory nitrogen source for Lemna and a good one for Spirodela. Ammonium ion was reported to be toxic to Lemna.

It is interesting that the wild-type Spirodela used in this work was partially heterotrophic when cultured on a completely defined, essentially inorganic medium. In its natural habitat, however, duckweed grows only in still water rich in organic materials and teeming with bacteria, which conceivably could supply a variety of factors. Whether or not the particular organic supplements that we have found to be stimulatory are the natural factors is, of course, open to question.

The growth-promoting effects of steroids on Spirodela have not been observed previously, although Withner and Rubin (22) reported that testosterone propionate stimulated growth of *Lemna minor*. Gorham (5) finds that the ether-soluble fraction from cow dung extracts stimulates growth of duckweed; this stimulation may likely be due to one or more steroids. Because this rather nonspecific stimulation by certain steroids was not discovered until our studies were well advanced, medium C was not replaced with a new basal medium containing a steroid. In future work with Spirodela on defined media, it would be well to include a steroid such as desoxycholic acid if optimum growth is desired. The role of steroids in plant metabolism is essentially unknown (7), and it is suggested that *Spirodela polyrhiza* represents a suitable biological material for research in this area.

Nature of the Auxotrophs: Since *Spirodela polyrhiza* has not been observed to flower in the laboratory, it was not possible to establish whether or not the new strains produced by X irradiation were mutants. These strains may be dominant mutants or propagatable chromosomal abberants resulting from translocations, inversions, or interstitial deletions. In any event, they have been maintained successfully by vegetative propagation in the laboratory with no evidence for reversion of the induced auxotrophy.

Among the varieties that were isolated, strain 301 showed the greatest difference in growth rate between medium C and medium D. Thus, strain 301 was chosen for the studies of nutritional requirements. Four other varieties also grew better in medium D than in medium C (table III). Among these, strain 349 was unusual in that it consistently grew better than the prototroph on medium B. Strain 262 showed an increased dependence on the supplements in medium B.

Table IV

Growth of Spirodela Polyrhiza Strain 301
Effects of Isoleucine and Leucine

Medium	Additions (mg/l)	Mean frond No.* after 7 days	Value of P from students t
C	...	12.9	...
D	...	17.8	< 0.01
C	L-leucine 4	15.1	0.10
C	L-isoleucine 4	15.8	0.05
C	L-isoleucine 4 + L-leucine 4	17.7	< 0.01

* Mean number of fronds from each of 12 replicate two-frond inocula.

Requirements of Strain 301: From the data obtained in this study, it is not possible to assess the real significance of the double requirement of strain 301 for L-leucine and L-isoleucine. This behavior may have arisen from two entirely separate biochemical defects induced by the X-ray irradiation. On the other hand, a great deal of information has been gained from studies in bacteria and yeasts (14) suggesting interrelationships between the three branched-chain amino acids, leucine, isoleucine, and valine. Thus, one may speculate that the requirement for both leucine and isoleucine may have resulted from a single biochemical lesion involving a common intermediate or similar types of chemical conversions.

No knowledge of biochemical pathways common to leucine and isoleucine has emerged from studies with microorganisms. In yeasts and bacteria, isoleucine and valine appear to be formed in closely related reaction sequences starting from α-ketobutyric and pyruvic acids, respectively (2, 15, 20, 21). Leucine appears to be derived from the isobutyryl portion of the

valine molecule (19). In spite of this possible relationship of leucine and isoleucine through valine, the growth of strain 301 was not promoted by L-valine alone or in combination with L-leucine or L-isoleucine. The results obtained here may imply that in Spirodela the pathways to leucine and isoleucine are closely tied together and that valine is formed by a separate pathway.

An alternative and possibly more likely explanation for the apparent double requirement of strain 301 for L-leucine and L-isoleucine could be based on a single block in the biosynthesis of L-leucine. A biochemical lesion just after L-valine should allow its accumulation. An excessive concentration of L-valine, in turn, might be expected to inhibit L-isoleucine utilization, causing an apparent additional growth requirement for L-isoleucine. This situation would be comparable to that in *E. coli* K-12 in which L-valine inhibits growth unless L-isoleucine is also present in the culture medium (12).

Summary

A wild-type *Spirodela polyrhiza* was isolated which for optimum growth required certain sugars (preferably glucose), choline, cystine, and gibberellic acid when cultured at 30° C under nearly continuous light at intensities of 400 and 2,000 foot-candles. This natural strain was also stimulated by adding certain steroids to the growth medium. Stigmasterol, ergosterol, and desoxycholic acid were particularly effective.

Five auxotrophic strains of *Spirodela polyrhiza* were produced by X-ray irradiation of the wild type. Growth of each of these new strains was stimulated by yeast extract. One strain (301) that failed to grow on a mineral salts medium which was adequate for growth of the original wild type was chosen for detailed study. The major growth requirements of strain 301 were determined. This strain required a mixture of L-isoleucine and L-leucine for optimum growth; neither amino acid alone was sufficient.

Acknowledgments

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ENHANCEMENT OF GIBBERELLIN GROWTH-PROMOTING ACTIVITY BY HYDRANGENOL ISOLATED FROM LEAVES OF HYDRANGEA MACROPHYLLA [1]

S. ASEN, H. M. CATHEY, AND N. W. STUART [2]

Hydrangenol (4′, 8-dihydroxy 3,4-dihydro isocoumarin)[3] was first isolated from the flowers of *Hydrangea hortensia* Smith, by Asahina and Miyake (1). Recently the authors isolated the aglycone and glucoside of hydrangenol from leaves of Engel's White hydrangeas. Coumarin and other unsaturated lactones are known to inhibit growth and germination (6, 14), but apparently there are no published accounts of the effect of the closely related isocoumarins on growth. The experiments reported here deal with the effect of hydrangenol in enhancing the growth-promoting activity of gibberellin.

Methods and Results

Characterization of Isolates From Engel's White Hydrangea Leaves: Two kg of fresh Engel's White hydrangea leaves from dormant plants stored at 4 to 5° C for 4 weeks were extracted by grinding with 80 % ethanol in a blendor. The aqueous ethanol extract was filtered through Hyflo Super-Cel and reduced to the aqueous phase by distillation in a vacuum. Chlorophyll was removed by filtration and the aqueous phase was extracted with ethyl ether in a liquid-liquid extractor for 2 weeks. After 48 hours of continuous extraction hydrangenol crystals were visible. Hydrangenol was removed from the ethyl ether and recrystallized three times from hot water. The yield was 2.4 g of the aglycone and 0.6 g of the glucoside of hydrangenol. The recrystallized hydrangenol was all converted to the aglycone by hydrolyzing with 2 N hydrochloric acid at 100° C under reflux for 1 hour.

The isolates from leaves of Engel's White hydrangeas were found to be indistinguishable from the glucoside and aglycone of authentic hydrangenol[4] (table I). Elemental analysis showed that the aglycone contained 70.24 % C, 4.67 % H, and 25.09 % O, whereas the theoretical values for $C_{15}H_{12}O_4$ are 70.28 % C, 4.72 % H, and 25.00 % O. The isolated glucoside yielded only glucose upon hydrolysis. The

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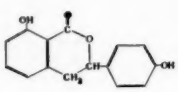
[3]



[4] The authors are indebted to R. K. Ibrahim, Botany Department, McGill University, Montreal 2, Canada, for a sample of authentic glucoside and aglycone of hydrangenol.

Table I

R_f Values for Authentic Hydrangenol (4′8-dihydroxy 3,4-dihydro isocoumarin) and Isolates from Leaves of Engel's White Hydrangeas

Solvent	R_f Values			
	Authentic hydrangenol		Isolates from leaves of Engel's White hydrangeas	
	Aglycone	Glucoside	Aglycone	Glucoside
n-Butyl alcohol: benzene: ammonium hydroxide (50:2:48 v/v)	0.59	0.15	0.58	0.15
n-Butyl alcohol: acetic acid: water (6:1:2 v/v)	0.96	0.71	0.96	0.71
n-Propyl alcohol: ammonium hydroxide (7:3 v/v)	0.85	0.47	0.85	0.46

isolated aglycone and glucoside both melted at 179° C (uncorrected), a value slightly lower than 181° to 182° C reported by Asahina and Miyake (1).

The ultraviolet absorption spectra of the isolates and the authentic aglycone and glucoside of hydrangenol were identical. In 95 % ethanol the aglycone and glucoside had absorption maxima of 315 and 300 mμ, respectively.

The cumulative evidence of paper chromatography, elemental analysis, melting point, and ultraviolet absorption spectra indicates that the compounds isolated from leaves of Engel's White hydrangeas are identical with the aglycone and glucoside of hydrangenol.

The aglycone of hydrangenol was used to study plant responses. This compound is sparingly soluble in water but soluble in dilute sodium hydroxide. A stock solution for treating plants was made by dissolving 1 mg of the aglycone of hydrangenol per 1 ml of hot water and adjusting the pH to 7 with sodium hydroxide. The final volume was adjusted to contain 0.1 % Tween-20.

ELONGATION OF MAIZE SHEATH: Seeds of d-1 mutant dwarf maize[5] were grown by the method described by Neely and Phinney (12). Seeds previously soaked in water for 24 hours were planted in soil in flats and then placed in a greenhouse with a minimum night temperature of 30° C. Approximately one week after planting, normal seedlings were removed and the dwarf ones just beginning to develop the first leaf were treated as shown in table II. Ten μl of the aglycone of hydrangenol and 10 μl of gibberellic acid (A_3), in appropriate concentrations, were placed separately at the base of the first sheath. The second leaf sheaths were measured to the nearest millimeter when they were fully expanded (12 days).

The aglycone of hydrangenol applied alone had little or no effect on the elongation of the second leaf sheath of d-1 mutant dwarf maize (table II). Elongation of the second leaf sheaths of d-1 mutant dwarf maize increased with increased concentrations of gibberellic acid applied to the plants. The response to 0.5 μg of gibberellic acid was increased by adding the aglycone of hydrangenol. Elongation of the second leaf sheath of d-1 mutant dwarf maize treated with a higher concentration of gibberellic acid (5 μg/plant) was increased by applying 0.1 μg of the aglycone of hydrangenol, but higher concentrations of the aglycone of hydrangenol tended to reduce the growth promotion of gibberellic acid.

In the previous test the solutions were applied to the same area of the first leaf sheath. To determine if the activity depended upon applying the chemicals to the same or separate areas, the aglycone of hydrangenol and gibberellic acid were applied from a mutual solution to the same area and from separate solutions to separate areas of the first leaf sheath of d-1 mutant dwarf maize. The total concentration of the surfactant applied to the plants was always constant. As shown in table III the aglycone of hydrangenol enhanced the growth-promoting activity of gibberellic acid to the same degree regardless of method of application.

[5] Seed supplied through the courtesy of B. O. Phinney, Department of Botany, University of California at Los Angeles.

TABLE II

MEAN LENGTH (mm) OF FULLY EXPANDED 2ND LEAF SHEATH OF d-1 MUTANT DWARF MAIZE TREATED WITH GIBBERELLIC ACID AND AGLYCONE OF HYDRANGENOL*

GIBBERELLIC ACID (μG/PLANT)	HYDRANGENOL AGLYCONE (μG/PLANT)				
	0.0	0.1	1.0	10.0	50.0
0.0	14	12	10	16	18
0.5	26	44	44	46	39
5.0	83	101	76	79	74

LSD 5 % = 14.7. 1 % = 22.8.
* Average for five plants per treatment.

TABLE III

MEAN LENGTH (mm) OF FULLY EXPANDED 2ND LEAF SHEATHS OF d-1 MUTANT MAIZE TREATED WITH GIBBERELLIC ACID AND AGLYCONE OF HYDRANGENOL ON SEPARATE AREAS FROM SEPARATE SOLUTIONS OR ON SAME AREA FROM A MUTUAL SOLUTION*

GIBBERELLIC ACID (μG/PLANT)	SOLUTION	HYDRANGENOL AGLYCONE (μG/PLANT)			
		0	0.01	0.1	1.0
0	...	22	25	25	21
0.1	Separate	40	49	49	46
0.1	Mutual	37	47	52	44

LSD 5 % = 4.6. 1 % = 7.3.
* Average for ten plants per treatment.

ELONGATION OF PEA AND BEAN: Seeds of *Pisum sativum* L. cultivar Meteor and *Phaseolus vulgaris* L. cultivar Black Valentine were soaked in water for 24 hours, planted in soil in 3-inch clay pots, and placed in a greenhouse at a minimum night temperature of 25° C. Approximately one week after planting, seedlings of uniform height were selected for treatment. Ten μl of the aglycone of hydrangenol and 10 μl of gibberellic acid in appropriate concentrations were placed separately on the leaves surrounding the growing point. The aglycone of hydrangenol was applied after the gibberellic acid solution was dry. Seedlings were measured to the nearest millimeter 1 week after treatment.

Gibberellic acid accelerated elongation of both Meteor pea and Black Valentine beans in relation to concentration. Gibberellic acid applied at the rate of 0.1 μg promoted extension of the second and third internodes of Meteor pea from 3.7 to 8.2 cm and Black

TABLE IV

MEAN LENGTH (cm) OF 2ND AND 3RD INTERNODES OF METEOR PEA 7 DAYS AFTER TREATMENT WITH GIBBERELLINS AND AGLYCONE OF HYDRANGENOL*

GIBBERELLINS		HYDRANGENOL AGLYCONE (μG/PLANT)			
TYPE	(μG/PLANT)	0.0	0.1	1.0	10.0
None	0.0	5.6	4.9	4.8	4.6
A_1	1.0	11.6	16.2	14.9	10.9
A_2	1.0	7.2	7.5	8.3	7.3
A_3	1.0	21.4	25.9	22.4	22.0
A_4	1.0	8.5	9.1	9.5	10.0

LSD 5 % = 2.4. 1 % = 3.8.
* Average for five plants per treatment.

Valentine bean from 9.2 to 16.3 cm. Applying 0.01 μg of the aglycone of hydrangenol plus 0.1 μg of gibberellic acid further increased the extension to 10.9 cm for the former and 22.3 cm for the latter[6]. Growth promotion by higher concentrations of gibberellic acid was not affected by applying the aglycone of hydrangenol.

GROWTH RESPONSES TO FOUR TYPES OF GIBBERELLIN AND AGLYCONE OF HYDRANGENOL: Seeds of Meteor peas were grown and the plants treated as previously described. Solutions of four pure gibberellins[7] (A_1, A_2, A_3, A_4) were prepared from warm distilled water containing 0.1 % Tween-20. Maximum growth responses of Meteor peas were obtained with A_3 followed in order by A_1, A_4, and A_2 (table IV). They are in agreement with the results reported by Bukovac and Wittwer (5).

Adding 0.1 μg per plant of the aglycone of hydrangenol significantly increased growth-promotion by gibberellin A_1 and A_3. Similar results were obtained with elongation of the second lateral peduncle of Shasta chrysanthemums.

DRY WEIGHT OF HYDRANGEA: Plants of *Hydrangea macrophylla* Ser. cultivar Sainte Therese were grown in an air-conditioned greenhouse at a mean temperature of approximately 22 to 25° C. The maximum temperature rarely exceeded 30°. Plants were treated weekly from June 4 until September 18 by spraying 1 ml of a solution containing 1 μg gibberellic acid and 1 μg of the aglycone of hydrangenol to the terminals of each plant. Plants were harvested September 24 and dried at 80° in a forced-draft oven. The values shown in table V indicate that applying the 16 μg of gibberellin resulted in significantly increased plant growth as reflected by stem extension and deposition of solids. The aglycone of hydrangenol alone exerted little effect but when combined with gibberellic acid it tended to enhance accumulation of total solids in the leaves and stems.

DISCUSSION

The action of gibberellin in promoting stem elongation and accumulation of total solids is well established (4, 7, 10, 17, 18). The present experiments show that a naturally occurring material, hydrangenol, increases the growth-promoting activity of low levels of exogenous gibberellin when applied to several test species. The material had little or no growth-stimulating activity alone and it failed to enhance the activity of higher levels of gibberellin. These results suggest that the aglycone of hydrangenol in some way affected the absorption or metabolism of gibberellin. Since the aglycone of hydrangenol increased the growth-promoting activity of gibberellic acid to the same degree whether applied from separate solutions to separate areas or from a mutual solution to the same area of the first leaf sheath of d-1 mutant dwarf maize, it must be affecting the cellular metabolism of the plant rather than enhancing the absorption of gibberellic acid.

Most plants have been postulated to contain naturally occurring gibberellins (16). Bean (15) and citrus (9) have been shown to contain A_1. Gibberellic acid, A_3, has not been isolated in a crystalline form from flowering plants. As shown the aglycone of hydrangenol alone was inactive but enhanced the growth-promoting activity of applied A_3 and A_1 much more than it did of the other two gibberellins. These results indicate that the aglycone of hydrangenol promoted primarily the activity of applied gibberellin, but did not affect the endogenous gibberellin.

The stem extension results suggest that the aglycone of hydrangenol is similar in action to the third factor postulated by Brian and Brian and Hemming (2, 3) and further expanded by Galston and Warburg (8) but interpreted by them as a sparing action of

TABLE V

GROWTH OF SAINTE THERESE HYDRANGEA PLANTS TREATED WEEKLY (JUNE 4 UNTIL SEPTEMBER 18) WITH 1 μG PER PLANT PER WEEK OF GIBBERELLIC ACID AND AGLYCONE OF HYDRANGENOL*

TREATMENT	PLANT HEIGHT CM	MEAN DRY WT/PLANT		
		STEMS G	LEAVES G	TOTAL G
Control-untreated	18.2	3.0	16.9	19.9
Hydrangenol aglycone	18.5	3.1	16.1	19.2
Gibberellic acid	36.6	5.2	19.1	24.3
Gibberellic acid + hydrangenol aglycone	36.7	5.8	21.2	27.0
LSD 5 % =	4.1	1.0	3.0	3.7
1 % =	5.5	1.3	4.0	5.0

* Average for ten plants per treatment.

[6] LSD at 5 %. Meteor peas = 1.4, Black Valentine bean = 2.2.

[7] Supplied through the courtesy of Yusuke Sumiki, Department of Agriculture Chemistry, Faculty of Agriculture, University of Tokyo, Tokyo, Japan.

auxin. The possibility must be considered that the aglycone of hydrangenol in some way exerted an auxin-sparing action which resulted in the increased growth. If this mechanism be accepted then one should expect that A: Application of the aglycone of hydrangenol alone should have enhanced the activity of gibberellins occurring naturally in the test plants and B: larger amounts of the aglycone of hydrangenol alone should have enhanced the activity of higher levels of applied gibberellin unless auxin became limiting. Neither of these actions was observed. However, Neumann (13), has reported that coumarin and several derivatives promoted the extension of small sections of Helianthus hypocotyls. Supra-optimal concentrations initially promoted extension but subsequently inhibited growth. Mayer (11) demonstrated that coumarin inhibited the action of gibberellin in promoting the germination of lettuce seeds. Both of these experiments and those which suggested the third factor were conducted with excised or isolated parts of the plant. Normal synthesis, translocation, and utilization of compounds was limited. This report deals with the intact plant where the complications of continuing growth preclude any simple explanation of the mechanism by which the aglycone of hydrangenol enhances the growth-promoting activity of applied gibberellin.

Summary

The aglycone and glucoside of hydrangenol (4'8-dihydroxy 3,4-dihydro isocoumarin) were isolated from leaves of Engel's White hydrangeas. The aglycone of hydrangenol by itself possessed little or no biological activity but when applied to several test species it enhanced the activity of low concentrations of applied gibberellin.

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SOME EFFECTS OF AMMONIA ON PLANT METABOLISM AND A POSSIBLE MECHANISM FOR AMMONIA TOXICITY[1, 2, 3, 4]

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Plant injury has been traced to ammonia derived from organic sources such as urine (9), chicken manure (8), cottonseed meal (12) and animal manure (3). In addition, inorganic sources such as aqua ammonia, anhydrous ammonia, and ammonium salts have been reported injurious to plants when placed too close to the root zone (3, 7). The toxicity of ammonia to animals has been demonstrated by Sumner (10). The injection of urease into rabbits resulted in death of the animals when NH_3 in the blood rose to 5 mg per 100 ml and before any change in blood pH could be detected. Ammonia and ammonium compounds are being used as fertilizers in increasing amounts. Therefore the question of how ammonia produces the toxic symptoms which frequently result from its use on plants becomes a matter of practical as well as theoretical interest.

The object of this work was to study the effect of ammonia on plant metabolism with the view of locating one or more sites at which ammonia could be shown to have a deleterious effect on the normal metabolic processes of plants.

MATERIALS AND METHODS

Respiratory rates of intact plant tissue and of mitochondria, in the presence and absence of ammonia and ammonium salt, were determined in a Warburg respirometer at 25° C.

Plant materials used included roots of barley, *Hordeum sativum* Pers., leaf and root disks of sugar beet, *Beta vulgaris* L. var. and garden beet. *B. vulgaris* L. var. spinach, *Spinacia oleracea;* and cells of *Chlorella pyrenoidosa* Chick.

The barley roots were grown in Hoagland's nutrient solution, cut when 20 to 30 mm in length, blotted dry, and treated with gaseous NH_3. Reproducible amounts of gaseous NH_3 were evolved from NH_4OH placed in the stopcock well of Dixon-Keilin flasks at 25° C. The molarity was calculated on the basis of one mole of gas occupying 22.414 liters under standard conditions. In other experiments leaves and root tissues and Chlorella were suspended in buffer and treated with solutions of ammonia. The undissociated ammonia concentration was calculated from the total concentrations of ammonium salt and the pH using a pKb for NH_4OH of 4.74.

Mitochondria were prepared from garden beet roots according to the method outlined by Honda et al (4) and modified slightly. All work was performed at 0 to 3° C with the equipment and solutions precooled at least 12 hours. Plant tissue was cut into approximately two centimeter cubes with a stainless steel knife. The homogenizing medium was a 1.0 M sucrose, with 0.2 M tris (hydroxymethyl) amino methane (tris), and 5×10^{-3} M ethylene diamine tetraacetic acid (EDTA) in redistilled water and adjusted to pH 8 with HCl. One hundred grams of the plant tissue with 100 ml of homogenizing medium were placed in a 1-liter Waring blender. The total blending time was 16 seconds in 4-second intervals 1 minute apart. The debris was removed with the aid of a Pexton laboratory press and the suspension centrifuged at 13,500 to 14,000 × G for 30 minutes to sedimate the mitochondria. The pellet was resuspended in 70 ml of a 0.6 M sucrose, EDTA, tris solution, and again centrifuged at 13,500 to 14,000 × G for 30 minutes. This washed mitochondria pellet was suspended at the rate of 4 ml of 0.6 M sucrose, EDTA, tris per 100 g fresh weight. It was necessary to force the final preparation through a glass wool pad in order to obtain a homogenous suspension. Aliquots of this preparation were taken for nitrogen determinations and subsequently analyzed by the micro-Kjeldahl method.

In mitochondrial studies involving substrate respiration the flask contained the following mixture: 2.0 ml of mitochondrial suspension, equivalent to approximately one milligram of N suspended in 0.6 M sucrose with 25 ppm terramycin according to Freebairn, et al (2), $MgSO_4$ and $MnSO_4$, 5 μmoles; adenosine diphosphate (ADP), 3 μmoles; diphosphopyridine nucleotide (DPN), 3 μmoles; substrate, 45 μmoles; and ammonium salts to provide an undissociated ammonia concentration of 4×10^{-3} M, (12 μmoles) or as indicated. Sufficient 0.2 M tris buffer was added to make a total volume of 3 ml. Results are reported in μl O_2 per hour per flask.

A Beckman Model DU spectrophotometer with a spectral energy recording attachment was used to follow the changes in absorbance of reduced diphos-

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[2] Aided by a grant from the American Cancer Society.

[3] Some of the data reported here are taken from the Ph.D. thesis of H. M. Vines, presented to the Graduate Division of the University of California at Los Angeles, August 1959.

[4] Paper No. 1217, University of California Citrus Experiment Station, Riverside.

[5] Present address: Citrus Experiment Station, Lake Alfred, Florida.

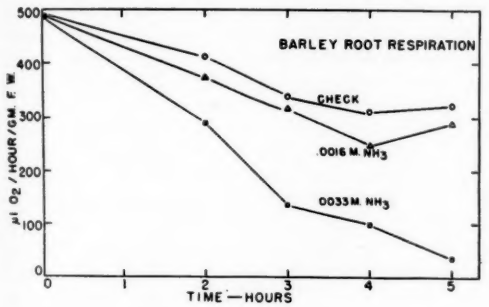
phopyridine nucleotide (DPNH) at 340 mμ (1) as it was oxidized and DPN as it was being reduced by beet root mitochondria. The conditions of these spectrophotometric observations are stated under the corresponding figures.

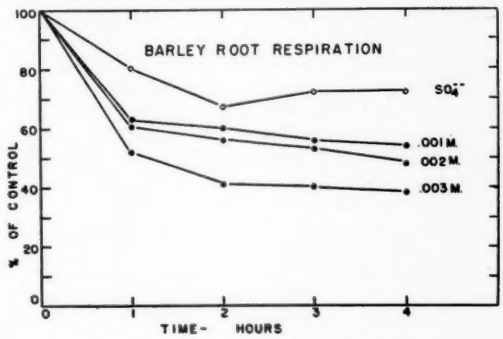
Results

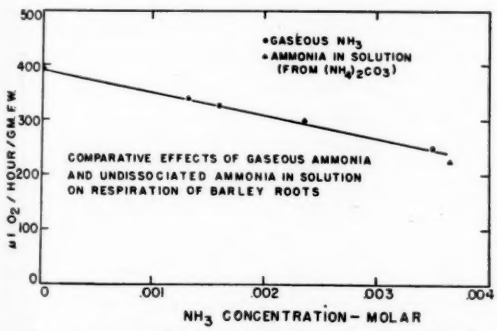
Ammonia Effects on Respiration of Excised Roots and Tissue Slices: A: *Inhibition of endogenous respiration.* Treatment of excised barley roots (blotted dry) with 1.6×10^{-3} M gaseous NH_3 in the flask atmosphere reduced their respiration 23 % and 3.3×10^{-3} M NH_3 caused a 78 % inhibition of respiration after 4 hours (fig 1). Although an effect of NH_3 on respiration of barley roots could be demonstrated in this way, some variability was encountered and the pH of the liquid film in contact with the roots could not be determined. In order to avoid this, solutions of ammonium salts were used. Concentrations of total ammonium salts equal to the gaseous ammonia did not give equivalent respiratory inhibition. Since the amount of inhibition due to ammonium salts was greater at higher pH, it appeared that the undissociated ammonia molecule might be primarily the form which penetrated the cells.

The respiration of excised barley roots treated with $(NH_4)_2SO_4$ solutions containing 1×10^{-3} to 3×10^{-3} M undissociated ammonia in 0.01 M phosphate buffer at pH 7 is shown in figure 2. These concentrations reduced the respiration rate 46 to 62 % within 4 hours after treatment. Although these solutions varied with respect to undissociated ammonia concentrations, they all contained the same concentration of the sulfate ion (0.28 M). The upper line of figure 2 shows that this concentration of sulfate alone inhibited the respiration of barley roots 28 % in 4 hours.

The effect of various other anions and cations on the respiration rate of barley roots at pH 8 was investigated. It was found that a greater inhibition was obtained when ammonia was supplied as the sulfate or chloride salt than when the anion was $CO_3^=$. Other monovalent cations such as K and Na had a






•GASEOUS NH3
▲AMMONIA IN SOLUTION (FROM (NH4)2CO3)
COMPARATIVE EFFECTS OF GASEOUS AMMONIA AND UNDISSOCIATED AMMONIA IN SOLUTION ON RESPIRATION OF BARLEY ROOTS
μl O2 / HOUR / GM. F.W.
NH3 CONCENTRATION - MOLAR

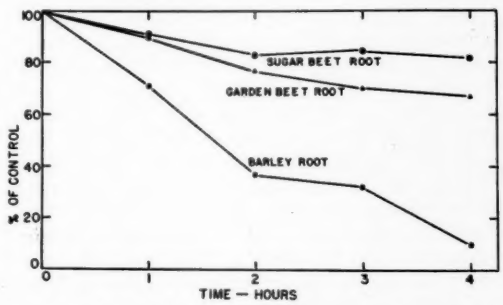


Fig. 1 (*upper left*). Effects of gaseous ammonia on the respiration rate of excised barley roots: △ = 1.6×10^{-3} M NH_3 evolved from 0.1 ml of 0.1 M NH_4OH; □ = 3.3×10^{-3} M evolved from 0.1 ml of 0.2 M NH_4OH; ○ = check.

Fig. 2 (*upper right*). Effect of the sulfate anion and undissociated ammonia on the respiration of excised barley roots. The control roots were in phosphate buffer at pH 7 and $SO_4^=$ was constant in all treatments at 0.28 M.

Fig. 3 (*lower left*). Comparison of the effects of gaseous ammonia and undissociated ammonia in solution on the respiration of excised barley roots at the end of a 2 hour exposure period. Calculation of undissociated ammonia was on the basis of final pH.

Fig. 4 (*lower right*). Effect of 4×10^{-3} M undissociated ammonia in solution at pH 7 on the respiration rate of disks cut from sugar beet roots and garden beet roots and excised barley roots.

slight depressing effect on barley root respiration for periods up to 2 hours, but after 4 hours the rates in the presence of these ions exceeded that of the controls, $NaHCO_3$ resulting in a 22% increase, for example.

Since it seemed possible that the undissociated ammonia molecule rather than the ion was effective in the inhibition of respiration, a direct comparison of the effects of undissociated ammonia in solution with gaseous NH_3 at similar concentrations on the respiration of barley roots was made. For this purpose the excised roots were treated either in the dry condition with an atmosphere containing NH_3 or suspended in buffer with ammonia supplied by $(NH_4)_2CO_3$ as described above. In figure 3 the inhibition of respiration by concentrations of these two forms of ammonia ranging from 1×10^{-3} M to 3×10^{-3} M after 2 hours exposure is shown. The undissociated ammonia in solution was calculated from the final pH at the end of the experiment. The maximum change in pH of the buffer due to a concentration of 0.7 M $(NH_4)_2CO_3$ was a rise from pH 8.0 to 8.26 at the end of 4 hours. These data show a close correlation between the inhibitory effect of undissociated ammonia in solution and NH_3 gas, lending support to the idea that both have a common form which is responsible for the inhibition.

The effect of ammonia upon respiration of garden beet and sugar beet root disks was similar to that on barley roots, although 4×10^{-3} M undissociated ammonia was somewhat less effective in inhibiting the respiration of the beet roots as is shown in figure 4.

B: *Inhibition of exogenous respiration.* The effect of ammonia on oxidation of glycolytic and Kreb's cycle intermediates was studied by infiltrating glucose, citrate, pyruvate, α-ketoglutarate or malate into root tissues. The infiltrated tissues, suspended in 3 ml of 0.2 M tris buffer at pH 8 were treated with ammonia by tipping in an appropriate concentration of $(NH_4)_2CO_3$ 20 minutes after closing the flasks. Figure 5 shows the increase in respiration of barley roots infiltrated with glucose, malate, and pyruvate, and the respiratory inhibition resulting from treatment of these roots with 3×10^{-3} M undissociated ammonia. In this experiment the initial control rate was 460 μl O_2/hr/g F.W. and at the end of 4 hours it was 310 μl O_2/hr/g F.W. In this and other experiments using $(NH_4)_2CO_3$, the first hourly readings for the ammonia treated flasks are omitted as it was found that approximately 30 minutes were required for manometric equilibration of the gases evolved when the ammonium carbonate solution was tipped into buffer containing no plant tissue.

It may be seen in figure 5 that ammonia has not only eliminated any exogenous respiration due to the added substrates, but has also inhibited endogenous respiration to about the same extent as was found in figure 2. Similar results were obtained with citrate and α-ketoglutarate.

In this experiment and in others in which substrates were infiltrated into beet root disks, beet leaf disks, and spinach leaf disks a respiratory response was not always obtained from the substrate, e.g., pyruvate in figure 5. In no case where a substrate gave a response was there any indication that it was being oxidized by a reaction located after a hypothetical metabolic block due to ammonia. To clarify this question and permit respiratory responses from other substrates, mitochondrial preparations were used in additional experiments described later.

Changes in Permeability Due to Ammonia: The inhibitory effect of ammonia on the respiration of excised roots or tissue slices could be due not only to some general or specific block in metabolism, but also might come about through an effect of ammonia on the integrity of the cell membrane, resulting in a partial disorganization of the cell reflected in a decreased oxygen uptake.

A: *Loss of anthocyanin from beet root disks.* The possibility of an effect on permeability was evaluated by measuring the loss of anthocyanin from garden beet root disks to the bathing medium under the influence of ammonia. Table I shows the change in absorbance of the bathing medium when 20 disks 9 mm in diameter and 1 mm thick were incubated in 10 ml of 0.2 M tris buffer at pH 8 for 2 hours in the

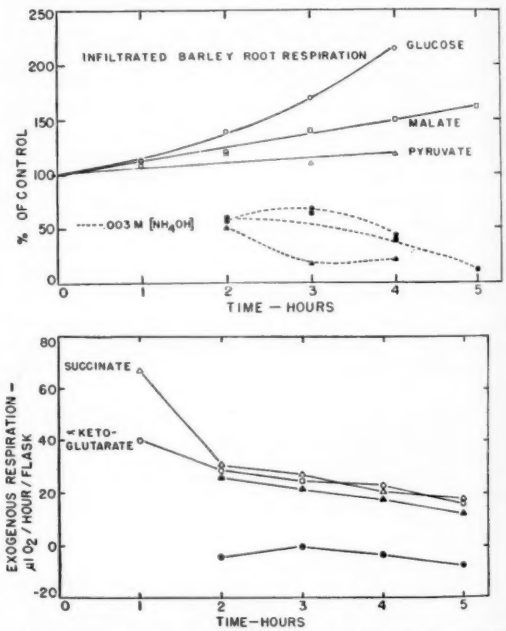


Fig. 5 (*upper*). Exogenous respiration rate of excised barley roots infiltrated with selected substrates at pH 8. Solid symbols indicate companion treatments treated with 3×10^{-3} M undissociated ammonia. Control was infiltrated with tris buffer.

Fig. 6 (*lower*). Rate of oxidation of succinate and α-ketoglutarate in garden beet root mitochondria (open symbols) and the effect of 4×10^{-3} M undissociated ammonia (solid symbols).

TABLE I

LOSS OF ANTHOCYANIN FROM RED BEET ROOT DISKS TO BATHING MEDIUM FOLLOWING TREATMENT WITH AMMONIA IN SOLUTION

UNDISSOCIATED AMMONIA	ABSORBANCE 410 mμ
Buffer	0.69
0.002 M	0.76
0.004 M	0.77
0.006 M	0.80
0.008 M	0.95
Boil	>2.00

presence of 0.002 M, 0.004 M, 0.006 M, and 0.008 M undissociated ammonia. One set of disks was boiled for 3 minutes to completely disrupt the cell membrane and provide a measure of the maximum possible loss.

It may be seen from this table that 4×10^{-3} M ammonia has increased the loss of anthocyanin from the beet disks by only 6 % of the possible maximum indicated by the boiled tissues, while the same concentration of ammonia caused more than 30 % inhibition of oxygen uptake (fig 2). Twice this concentration of ammonia has brought the loss of anthocyanin up to 20 % of the possible maximum, but damage to cell membranes seems inadequate to explain the observed effects on respiration.

B: *Loss of S^{35} labeled material from Chlorella.* In another attempt to evaluate the effects of ammonia on permeability, Chlorella cells were labeled with S^{35} by being incubated for 4 hours in nutrient solution containing 0.28 M $S^{35}O_4^{=}$. They were washed twice by centrifugation to remove external isotope and incubated for 2 hours with various concentrations of ammonium salt in 0.2 M tris buffer at pH 8. The amount of S^{35} in both the cells and the bathing medium was determined. 4×10^{-3} M ammonia has resulted in even less loss of S^{35} labeled material from the cells than was found in buffer alone (table II).

AMMONIA EFFECTS ON OXIDATIVE ACTIVITY OF MITOCHONDRIA: In order to minimize the problems caused by the presence of a permeability barrier at the cell membrane and to facilitate study of ammonia effects on the oxidation of individual substrates, treatments of garden beet mitochondria were undertaken.

TABLE II

LOSS OF $S^{35}O_4^{=}$ TO BATHING MEDIUM FROM CHLORELLA PYRENOIDOSA INCUBATED FOR 2 HOURS WITH ISOTOPE THEN TREATED FOR 2 HOURS WITH AMMONIA IN SOLUTION

UNDISSOCIATED AMMONIA	% S^{35} EXTRACTION
Buffer	14.7
0.002 M	5.7
0.004 M	6.5
0.008 M	17.8
Boil	75.2

The effect of external pH on the response of mitochondria to ammonia was similar to that observed with intact cells, and indicated that the molecular form of ammonia was also the effective inhibiting agent here.

A comparison of carbonate, sulfate, and phosphate salts of ammonia on the oxidative activity of beet mitochondria showed that equal concentrations of $(NH_4)_2SO_4$ were more toxic than either NH_4CO_3 or $(NH_4)_2HPO_4$ and the carbonate salt was used in mitochondrial studies.

Figure 5 summarizes the effect of 3×10^{-3} M undissociated ammonia on the oxidation of glucose, pyruvate, citrate, and malate by beet root mitochondria where the exogenous respiration of these compounds is entirely eliminated by ammonia within 2 hours.

Similar data for the respiration of α-ketoglutarate and succinate by garden beet root mitochondria are given in figure 6 which shows that although α-ketoglutarate oxidation is completely suppressed by 4×10^{-3} M undissociated ammonia, this same concentration has relatively little effect on succinate oxidation for periods up to 5 hours.

This partial resistance of succinate oxidation to ammonia inhibition seemed to point toward a possible association of DPN with ammonia toxicity, since DPN does not serve as a cofactor for succinate oxidation (5). A study of the effect of ammonia on the oxidation and reduction of DPN by beet homogenates showed that DPNH oxidation was completely inhibited while no effect was found on the reduction step (11). Since the homogenates contain soluble enzymes and cofactors, and high concentrations of ammonia were supplied as a gas, it was desirable to study the reactions under more controlled conditions.

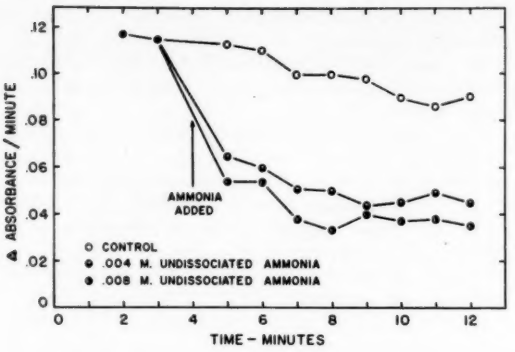
Mitochondria prepared from garden beet roots were used for this purpose. The mitochondria were washed twice in 0.6 M sucrose in 0.2 M tris buffer at pH 8 after being prepared in 1.0 M sucrose with 5×10^{-3} M EDTA and 0.2 M tris buffer at pH 8.

Figure 7 shows the rate of DPNH oxidation by these mitochondria plotted as △ absorbance/minute over a 12-minute period and the effect of 4×10^{-3} M and 8×10^{-3} M undissociated ammonia on this oxidation. The lower concentration caused an inhibition of about 50 % and doubling the concentration increased this inhibition somewhat.

Similar data for the reduction of DPN by mitochondria supplied with citrate and treated with ammonia are given in figure 8. The slight, but significant increase in the rate of DPN reduction caused by ammonia may be related to the inhibition of the reverse reaction, and has not been consistently found in all similar experiments.

DISCUSSION

Because of the alkaline nature of ammonia in solution the pH per se has often been implicated as the basic cause of toxicity to plants resulting from ammonia applications. However, the results of this study indicate that the toxicity results from reactions



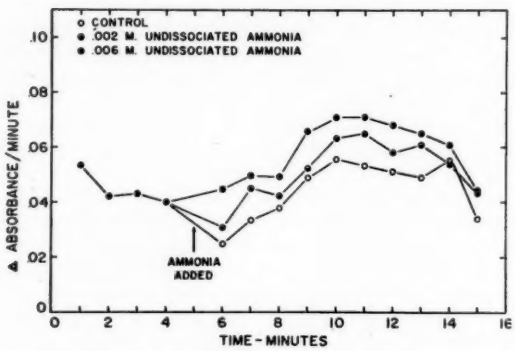


FIG. 7 (*upper*). Effect of ammonia on the rate of oxidation of DPNH in mitochondria plotted as change in absorbance per minute at 340 mμ at pH 8. Each cuvette contained cytochrome c, 0.225 mg; DPNH, 3 μmoles; mitochondria 0.5 ml; appropriate ammonia or buffer and sufficient 0.2 M tris at pH 8 to make a total volume of 3 ml.

FIG. 8 (*lower*). Effect of ammonia on the rate of reduction of DPN in mitochondria plotted as change in absorbance per minute at 340 mμ at pH 8. Each curvette contained DPN, 3 μmoles; citrate, 30 μmoles, appropriate ammonia, or buffer and sufficient 0.2 M tris at pH 8 to make 3 ml.

of ammonia inside the cells and that the primary effect of a high pH in increasing damage to plants from ammonia applied to the soil is probably to cause an increase in the amount of ammonia entering the cell.

The form of ammonia which is an effective inhibitor of respiration in excised plant tissues and mitochondria appears to be the undissociated molecule rather than the ammonium ion. An equivalent concentration of ammonia gas and undissociated ammonia in solution produced the same inhibition of respiration in excised barley roots. With other leaf and root tissues an increased respiratory inhibition was found when the pH of a buffer containing a constant concentration of ammonium salt was raised. If the same concentration of undissociated ammonia is maintained by reducing the pH and increasing the concentration of ammonium salt, the inhibitory effect remains at the same level in either tissue preparations or mitochondria. Whether this relation arises through failure of the ions to penetrate the permeability barriers of cells or particles or whether the molecule itself is active at the site of inhibition cannot be determined from the data available.

The possibility that ammonia was producing inhibitory effects on tissue respiration through a nonspecific effect on permeability of the cell membranes and thus resulting in a loss of organization of the protoplasm, was largely eliminated by demonstration of the effect of ammonia on the permeability of cells was much less than its effect on respiration. However, the possibility of some more specific effect of ammonia on cell permeability remains, and the effect on permeability of mitochondria has not been investigated.

The evidence presented on the inhibition of the respiration of intact cells by ammonia indicates that ammonia blocks metabolism but there is no suggestion as to the location of the block. There is some indication that a fraction of endogenous respiration proceeds by a pathway which is resistant to ammonia inhibition, particularly in beet root disks and spinach leaf disks. The exogenous respiration of glucose, citrate, and malate by barley roots and other plant tissues was inhibited by ammonia in the same way as the endogenous respiration.

These substrates as well as pyruvate, α-ketoglutarate, and succinate were found to give respiratory responses with mitochondrial preparations, but oxidation of all these substrates except succinate was inhibited by ammonia.

Succinate oxidation is known to proceed without the utilization of DPN, although the remainder of the electron transport system is common to succinate and other Kreb's cycle intermediates (1). This fact, coupled with the resistance of succinate oxidation to ammonia treatment indicated that ammonia toxicity might be associated with reduction or oxidation of DPN. Inhibition of DPNH oxidation by ammonia in homogenates has been reported by Wedding et al (11).

The oxidation of DPNH by mitochondria from beet roots was found to be inhibited by either gaseous NH_3 or ammonia supplied in solution via ammonium salts. The reduction of DPN shows either no response to ammonia, or a slight stimulation which may be due to simultaneous inhibition of the reverse reaction.

Work recently reported by Krogmann et al (6) using spinach chloroplast preparations indicated that ammonia is an effective uncoupler of photosynthetic phosphorylation. This may represent a similar interference of ammonia with electron transport in the photosynthetic reaction.

It is suggested that at least part of the toxicity of ammonia is due to its ability to specifically inhibit the oxidation of DPNH and thus block the transport of electrons from oxidized substrates to oxygen.

Summary

The mechanism of ammonia toxicity to plants was investigated. Oxygen uptake in a Warburg respirometer was used as an indication of the effect of gaseous ammonia and ammonium salts on respiration of excised barley roots, garden beet root disks, as well as leaf disks of spinach and sugar beets, and garden beet root mitochondria.

Gaseous NH_3 and undissociated ammonia in equal concentrations were found to inhibit respiration to the same degree in the tissue studied. The pH level was important in ammonia toxicity to the extent that it controlled the undissociated ammonia concentration. Therefore the undissociated ammonia probably is the effective form of ammonia causing inhibition of respiration in plants.

Added substrates were ineffective in overcoming the respiratory inhibition of infiltrated intact tissue or mitochondrial preparations. Substrates studied include glucose, pyruvate, citrate, alpha-ketoglutarate, succinate, and malate. Of these only succinate appeared resistant to ammonia.

The partial resistance of succinate oxidation to ammonia treatment indicated a possible association with the electron transport system since succinate does not require diphosphopyridine nucleotide (DPN) as a cofactor. Studies with DPN reduction and oxidation in a recording spectrophotometer at 340 mμ showed that the oxidation of reduced diphosphopyridine nucleotide (DPNH) was inhibited by ammonia treatment. The companion reaction, DPN reduction, was speeded up following ammonia treatment, probably due to the competition of the two simultaneous reactions.

It is suggested that the site of ammonia toxicity to plants is located in the electron transport system, specifically on the DPNH→DPN reaction.

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ACCUMULATION OF POTASSIUM, CESIUM^{137}, AND RUBIDIUM^{86} IN BEAN PLANTS GROWN IN NUTRIENT SOLUTIONS[1, 2]

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In evaluating uptake into the biosphere of Cs^{137} from fallout, frequent use has been made of a Cs^{137}/K ratio (4, 10, 11). A similar ratio of Sr^{90}/Ca has assumed importance in interpreting data on the uptake of Sr^{90} (9, 13). The validity of the Cs^{137}/K has been assumed from relatively small amounts of experimental data on the interaction of these two elements (1, 3, 11). Use of such ratios to evaluate hazards has been questioned by Kornberg (7, 8) on the basis of data obtained in this laboratory with isotopically labeled ion pairs.

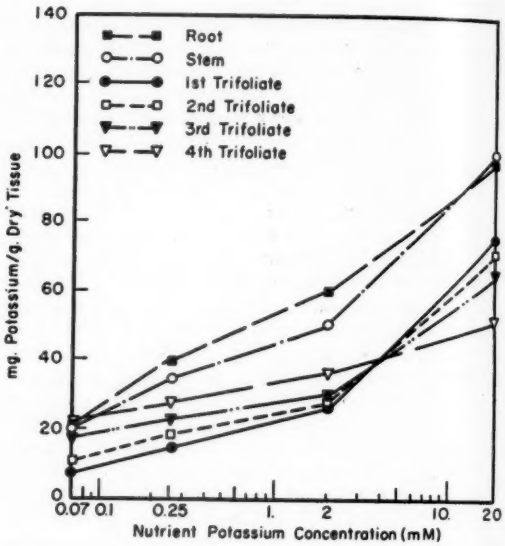
Results described in this report deal with the simultaneous uptake of Cs^{137} and potassium by plants. The results of similar experiments with Rb^{86} and potassium are also reported.

METHODS

Phaseolus vulgaris L. were grown by nutrient culture techniques in controlled environment growth chambers (12). The nutrient solution was prepared by a modified formula of Johnson et al (6). The macronutrient concentrations were: 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 1 mM $(NH_4)_2HPO_4$, and 0.5 mM $MgSO_4$. Potassium was adjusted to concentrations from 0.07 to 20 mM by adding KNO_3. Total nitrogen was held constant by adding NH_4NO_3. Micronutrients added were: Cl, B, Mn, Fe (added separately), Zn, Cu, and Mo at respective concentrations of 1.77, 0.27, 0.27, 0.22, 0.13, 0.03, and 0.01 ppm.

Cesium 137 was added uniformly to all culture media at a concentration of 0.8 microcuries per liter; the Rb^{86} concentration was 0.4 microcurie per liter. Carrier cesium was added at concentrations of 0.02 or 0.2 mM (table I), and stable rubidium was 0.003 mM. Each treatment was triplicated with six plants per replicate. Seven-day-old seedlings were placed in the test solution and allowed to grow for 16 days. Nutrients were replaced every 3rd day with fresh solutions containing the initial concentrations of nutrients and radioisotopes. The pH was maintained between 6.0 and 6.3.

Plants were divided for analysis into stems, roots, and leaves with the latter divided according to age for some assays. Roots were blotted dry to remove



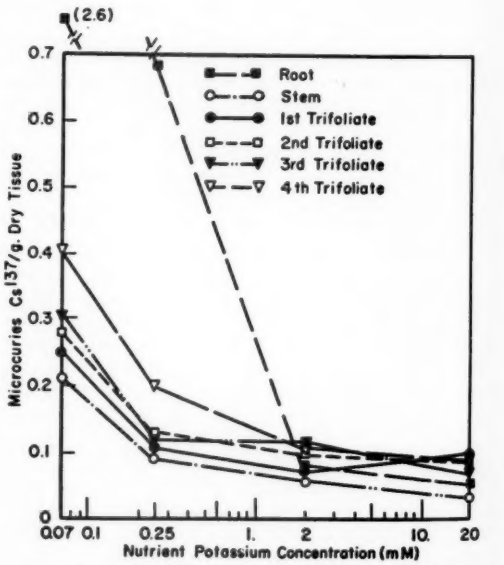


FIG. 1. Accumulation of potassium in plant tissue.
FIG. 2. Effect of increasing nutrient potassium on Cs^{137} concentration in bean plants.

[1] Received March 31, 1960.

[2] Work performed under Contract No. AT(45-1)-1350 between the Atomic Energy Commission and the General Electric Co.

excess nutrient solution. Samples were dried in an oven at 70° C for 48 hours, weighed, and digested in hot nitric acid. Digests were not permitted to go to dryness since some cesium salts decompose at relatively low temperatures which could result in loss of Cs^{137}. The digested materials were brought to volume, and aliquots plated and assayed for Cs^{137} or Rb^{86} using the thin mica end-window beta counters. Potassium concentrations were determined on the same digests with a Perkin-Elmer, Model 146, flame photometer using the lithium internal standard method described in the manufacturer's operating manual.

Results

By comparing figures 1 and 2, it is clear the concentrations of both potassium and cesium in the plants were influenced by external concentrations of potassium. With progressively increasing amounts of externally supplied potassium, the potassium content of the leaves increased while the Cs^{137} content decreased. The decrease in Cs^{137} content was most significant in young leaves while the largest potassium increase was in older leaves. Rates of change were greater at the lower potassium concentrations.

Potassium concentrations in roots and stems were generally greater than in leaf tissues. This difference was accentuated with higher concentrations of potassium in the substrate. There was less Cs^{137} in stems than in other tissues at all potassium levels. The Cs^{137} content of roots was exceedingly high at the low potassium levels, but with substrate potassium concentrations of 2 mM and higher, Cs^{137} concentrations were higher in leaves than in or on roots.

Dry weights of the various plant parts were maximum with 2 mM potassium in the substrate (table I). A toxic effect of cesium was clearly expressed through decreased dry weight, yellow older leaves, and abnormal root growth when 0.2 mM cesium and 0.07 mM potassium were in the substrate. Such a plant is shown on the left side of figure 3, but unfortunately the abundant adventitious roots, characteristic of cesium toxicity, are not clear in this photograph.

FIG. 3. Cesium toxicity symptoms after 16 days exposure to treatment.

Toxicity was not due to depressed potassium uptake. Data in parenthesis in table I show that those plants which were damaged contained at least as high a concentration of potassium in the leaves compared to those grown on less cesium. The lower potassium in roots and stems suggests that its distribution may be modified even though uptake is unaffected.

Data reported here are from experiments in which potassium was added as the nitrate with total nitrogen held constant in all cultures by adding ammonium nitrate. Corresponding experiments were also conducted using KCl with nitrogen supplied uniformly

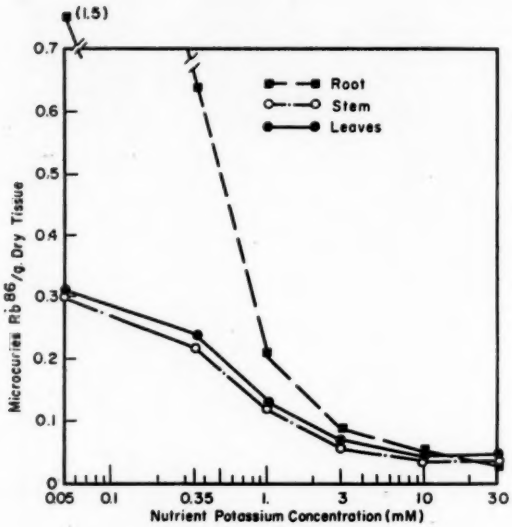
TABLE I

WEIGHT AND POTASSIUM CONTENT OF PLANTS AS FUNCTION OF POTASSIUM CONCENTRATION

SUBSTRATE CONCENTRATIONS mM		GRAMS DRY WEIGHT (MG K/G DRY TISSUE SHOWN IN PARENTHESIS)					
K	Cs	ROOTS	STEMS	PRIMARY LEAVES	1ST & 2ND TRIF	3RD & 4TH TRIF	TOTAL PLANT
0.07	0.02	1.02	0.94	0.70	1.25	1.25	5.21
		(21)	(21)	(12)	(10)	(21)	(17)
0.25	0.02	1.47	1.43	0.89	1.68	1.82	7.30
		(40)	(35)	(19)	(18)	(26)	(28)
2.0	0.02	1.58	1.75	0.98	1.68	2.13	8.11
20.0	0.02	1.58	1.74	0.95	1.74	1.51	7.53
0.07	0.2	0.71	0.81	0.82	0.84	0.77	3.95
		(12)	(14)	(14)	(17)	(22)	(16)

to all cultures in concentrations according to the basic formula. Under both conditions results were similar, indicating little or no interference by the chloride, ammonium, or nitrate ions.

Results from similar experiments using Rb^{86} instead of Cs^{137} are shown in figure 4. Plant concentrations of potassium were essentially as in figure 1 and are thus not shown. As with Cs^{137}, the concentration of Rb^{86} in the various plant parts decreased as concentrations of potassium in the substrate and plants increased. The considerably higher Rb^{86} content of the roots as compared to stems and leaves at the lower potassium nutrient levels, was also similar to that obtained with Cs^{137}.



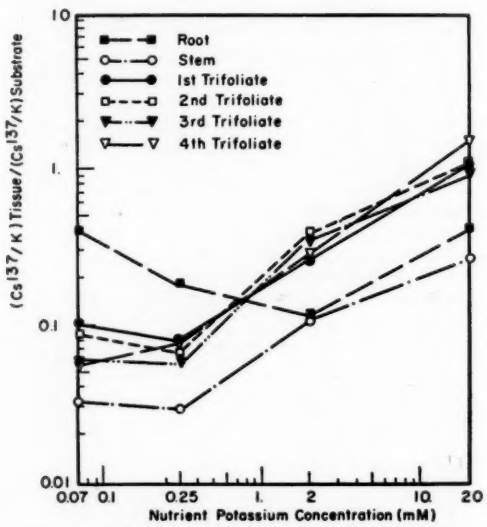


FIG. 4. Effect of increasing nutrient potassium on the uptake of Rb^{86} by plants.

FIG. 5. Effect of increasing potassium on DF values for Cs^{137}/K.

DISCUSSION

The study of differential uptake by plants of cesium and potassium was facilitated by following the ratio of cesium to potassium in the plant tissues relative to the ratio in the substrate. We have chosen to use the term "discrimination factor" (DF), i.e., (Cs^{137}/K) plant/(Cs^{137}/K) substrate, because it is descriptive and has previously been defined (*2*). Values greater than one indicate discrimination against potassium, and values less than one indicate discrimination against cesium. This term and similar expressions (observed ratio and distribution factor) have been used by other investigators (*2, 4, 11*) to compare concentrations in the organism with those in the substrate. The same value is obtained if one divides the percentage of Cs^{137} taken up by the percentage of potassium taken up.

In figure 5, the DF is plotted as a function of substrate potassium. It is clear that the degree of discrimination between Cs^{137} and potassium in aerial parts is not uniform. For plant parts other than roots, the DF rises about tenfold with a 200-fold increase in substrate potassium. The rise in DF is tempered by the increased potassium uptake over the range of substrate concentrations studied. However, the combined increase of potassium and decrease of Cs^{137} is not great enough to keep the ratio of these in the plant comparable with the ratio in the substrate. Thus, it seems that the uptake of these two ions is in part non-competitive. These results appear to disagree with those of Collander (*1*). This may be a consequence of the wider range of concentrations used in the present study.

Figures 1 and 2 show that potassium and cesium concentrations of leaves varied with age or position on the plant. The position relationship was changed by varying the potassium in the nutrient solution. The young top leaves of plants grown in low nutrient potassium contained higher potassium and cesium concentrations than did lower and older leaves. When nutrient potassium became excessive, a reverse relationship was observed with the higher potassium concentration in the older leaves. This same reversal was not noted for cesium.

This reversal with increasing potassium is easily explained on the basis of a relationship between interleaf transport and availability of freshly absorbed material from the root. If abundant potassium is available from roots, little translocation of this element from old to new tissue is observed. It can even be visualized as a detoxification process with all cells continuing to deposit excess potassium in their vacuolar spaces and thus building the concentration in older tissues. When potassium is limiting, it is translocated from existing tissue to supply demands for

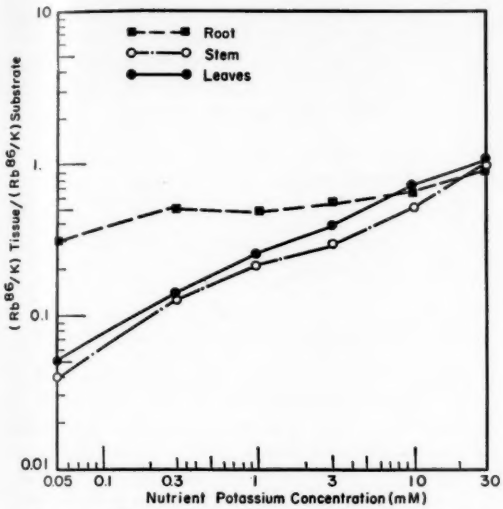


FIG. 6. Effect of increasing substrate potassium on DF values for Rb^{86}/K.

growth as suggested by Janssen (5). This interpretation also correlates well with the tissue studies of Steward and Millar (14) and Sutcliffe (15) who observed a higher concentration of Cs^{137} in vacuolated non-growing cells than in growing cells of carrot.

The relationships between Rb^{86} and potassium were similar to those between Cs^{137} and potassium. This is most easily seen by comparing figures 5 and 6. Although the DF factors for the two pairs are similar at the potassium concentration tested, there is less evidence of intra-plant selection between Rb-K than between Cs-K. This can be concluded from figure 6 by the closeness of the stem and leaf values.

It is important to note that the efficiency of uptake, i.e., DF value, for Cs^{137} with respect to potassium, is not uniform. Failure of the plant to take up these ions in a fixed proportion to their concentrations in the substrate means that prediction of Cs^{137} concentration in the biosphere on the basis of a Cs^{137}/K ratio in the soil is potentially in error. The fact that soil tends to buffer against extremes in concentration of ions available for uptake may well prevent the observation of the extreme changes in DF values as noted here. Precise predictions of plant uptake of Cs^{137} can be achieved only by evaluating isotope uptake from important soils in a given area.

SUMMARY

Adding potassium to the nutrient substrate reduced Cs^{137} and Rb^{86} uptake by bean plants less than expected from an assumed physiological equivalence of these ions. Plants discriminated against cesium at low potassium nutrient concentrations, but with increasing substrate potassium, this discrimination diminished. Discrimination of Rb^{86} from potassium approximated that observed for Cs^{137}. Potential errors from the use of ratios in predicting uptake of Cs^{137} were discussed.

Some toxicity was noted when significant quantities of non-radioactive cesium were in the nutrient solution.

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PATHWAYS OF GLUCOSE DISSIMILATION IN CARROT SLICES[1, 2]

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There is now ample evidence that the pentose phosphate (PP) pathway contributes to the breakdown of glucose in many plant tissues (2, 5). We are ignorant of the precise significance of this sequence and of its exact relationship to the other main route of glucose catabolism in higher plants, the Embden-Meyerhof-Parnas (EMP) sequence. The likelihood of close interplay between the two pathways is recognized since some intermediates and enzymes are common to both. One result of this is the possibility that triose phosphate produced from pentose in the PP pathway is converted to pyruvate by enzymes normally regarded as belonging to the EMP sequence. The extensive metabolism of pentose, which would result from these reactions, and which is commonly overlooked in calculating the contributions of the two pathways, now appears to be of some importance in plant metabolism. Viewed in this way the two sequences differ only in the reactions by which they produce triose phosphate. In the EMP sequence triose phosphates are the only products but in the PP pathway the generation of each mole of triose phosphate is accompanied the reduction of 6 moles of triphosphopyridine nucleotide (TPN), the release of 3 moles of CO_2 from C-1 and the regeneration of 2 moles of hexose.

In the work reported in this paper we have examined the interactions of the two pathways in carrot tissue. Carrots were chosen because there is already good evidence that glucose breakdown occurs by both pathways (6, 14, 21). In the first group of experiments, attempts were made to alter the balance between the two pathways by using the following selective agents, arsenite, 2,4-dinitrophenol (DNP), methylene blue, and iodoacetate. The relative rates of release of carbons 1 and 6 of supplied glucose was the criterion studied in this series of experiments. The relative importance of the two pathways in untreated tissue was investigated next by A: measuring the progress of the release of C^{14} as $C^{14}O_2$ from specifically labeled glucoses and pyruvates over long periods and B: determining the contributions of individual carbons of the glucose to other cellular components.

MATERIALS AND METHODS

Carrots of varied origin were bought at the local store. They were kept at 5° C and used within 4 weeks. Very young carrots were not used. Cylinders of tissue, comprising both phloem and xylem, were removed from the tap roots with a No. 4 cork borer and cut into sections roughly ¾ mm thick. The discs were cut into distilled water and then rinsed twice, dried on paper towels, and divided into random samples by careful weighing. These samples were used at once without further washing. These preparations were completed within half an hour.

In all experiments the tissue was placed in 0.03 M potassium phosphate at pH 5.0, 3.0 ml/g fresh weight of tissue, and maintained at 25° C. Measurements of gas exchange were carried out by standard Warburg techniques on 650 mg samples of tissue. Respiratory inhibitors were dissolved in 0.03 M phosphate and the pH adjusted to 5.0. These solutions were prepared immediately before use.

Uniformly labeled glucose and glucose-1-C^{14},-2-C^{14} and -6-C^{14} were obtained commercially. Glucose 3,4-C^{14} was isolated from 5 day old castor beans which had been exposed to $C^{14}O_2$ in the dark for 5 minutes. Under these conditions 90 % of the carbon^{14} incorporated into glucose is confined to carbon atoms 3 and 4 (19). Pyruvate-1-C^{14}, -2-C^{14}, -3-C^{14} were obtained from the Radiochemical Center, Amersham, U.K.

In the experiments with labeled substrates 2 μmoles of the appropriate glucose, or pyruvate, were added to 1.3 g of tissue in 4.0 ml of buffer, in Warburg vessels with a volume of approximately 120 ml. Glucose uptake was estimated by measuring the radioactivity of samples (0.2 ml) which were withdrawn from the external medium at regular intervals.

The $C^{14}O_2$ respired by the tissues was absorbed by KOH in the center wells. At intervals the KOH was removed quantitatively and replaced by fresh alkali. The carbonate present in each sample of KOH was precipitated as barium carbonate and counted. C_6/C_1 ratios were determined as described by Beevers and Gibbs (7). The measurement of the effects of respiratory inhibitors upon the release of $C^{14}O_2$ was complicated by their inhibition of glu-

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cose uptake. The final concentrations of arsenite and 2,4-dinitrophenol (DNP) chosen almost completely inhibited glucose uptake. In the experiments with these inhibitors the tissue was first supplied with glucose for 3 to 4 hours in the absence of the inhibitors. The samples were then removed from the glucose, quickly washed with buffer and placed in solutions of the appropriate inhibitor. The concentrations of methylene blue and iodoacetate chosen caused only slight inhibition of glucose uptake. Therefore these compounds were added to the tissue with the labeled glucose. Appropriate controls were always run. The values in the tables and graphs are the averages of duplicate, or in most experiments, triplicate determinations. The results were further confirmed by repeating the individual experiments with different batches of carrots.

FRACTIONATION OF TISSUE: After removing the external solution, boiling 80 % ethanol was added to the tissue in the Warburg flasks. The tissue was then extracted in a Soxhlet apparatus with 200 ml of 80 % ethanol at 45°C for 48 hours. The ethanol was distilled off and the aqueous solution extracted with a few milliliters of hexane to remove pigments. This aqueous solution was then fractionated into amino acids, organic acids, and sugars by passage through columns of ion exchange resins as described by Neal and Beevers (15).

The fraction of the tissue which was insoluble in 80 % ethanol, was dried to a constant weight in a vacuum oven at 50° C. Carefully weighed aliquots were combusted to give an estimate of the total C^{14} incorporated into this fraction. The remainder of the fraction, roughly 80 %, was hydrolysed by heating in a sealed tube with water and five times its own weight of Dowex 50 WX10 resin at 100° C for 50 hours. The contents of the tube were then transferred to a column of the same resin in the acid form and the whole thoroughly washed with water. The effluent contained the carbohydrates and the acids. The resin was then eluted with 1 N ammonium hydroxide to give the amino acids. In all this work solvents were removed and solutions were concentrated by boiling under reduced pressure at temperatures below 40° C.

Each of these main fractions was separated into its major components by paper chromatography. Chromatograms were always prepared in duplicate. The amino acids were separated by two directional chromatography on Whatman No. 20 paper. Phenol saturated with water was used as the first solvent and butanol-formic acid-water (4:1:5) as the second. The organic acids and the sugars were separated on Whatman No. 1 paper. Butanol-formic acid-water (4:1:5) was used to separate the acids. Ethyl acetate-acetic acid-water (3:1:2) was used to separate the sugars.

MEASUREMENTS OF RADIOACTIVITY: C^{14} was measured by counting in a windowless gas flow counter. Samples were prepared in two ways. In the first, 0.2 ml of the radioactive solution was dried gently in the center of a nickel planchette. This procedure was found to be unsatisfactory when a truly quantitative comparison of the activity of different fractions of a tissue extract was required. Presumably, differences in the amounts of solutes present in each fraction led to significant variations in the self absorption of the samples prepared for counting. This method was used only in the estimation of glucose uptake and in the assessment of activity present in eluates of paper chromatograms. All other measurements of the activity of C^{14} were made after it had been converted to barium carbonate. Radioactive compounds and solutions were oxidized to CO_2 by wet combustion and the CO_2 subsequently precipitated as barium carbonate. The barium carbonate was counted as an even layer spread over the surface of a porous micro-filter. These measurements were corrected for self-absorption of the carbonate.

Radioautograms of chromatograms of amino acids were prepared. The distribution of label on all other chromatograms was determined both by preparation of radioautograms and by elution of individual components followed by estimation of the activity present in each eluate. Compounds were located on chromatograms by the development of a strip on which both authentic and the unknown compounds had been run.

RESULTS

The initial rates of respiration of the carrot slices used in these experiments were similar from day to day and averaged 83 microliters O_2/g fresh weight/hour. Respiratory quotients were frequently determined and were always found to be close to 1.0 Adding glucose at the concentrations used in the main experiments did not lead to any detectable change in the rate of O_2 uptake or in the RQ. The absorption of micromolar amounts of glucose by slices of the kind used here is discussed in a second paper (1). The rate of uptake increased with the development of the induced respiration until the glucose was completely absorbed after about eight hours.

EFFECTS OF INHIBITORS AND OTHER AGENTS ON C_6/C_1 RATIO: The initial rates of production of $C^{14}O_2$ from glucose-6-C^{14} and glucose-1-C^{14} were determined and confirmed a previous observation (7) the ratio between these values was close to 0.50, indicating that some glucose was being catabolized by the P.P. sequence. Over a fourfold range of glucose concentrations (0.5–2.0 μmoles/ml) the C_6/C_1 ratios remained constant. It was hoped that by applying appropriate amounts of suitable reagents whose action is more or less specific, a predictable change in the relative participation of the PP and EMP pathways might be brought about.

I. Experiments with arsenite. It was first established that arsenite, which is known to prevent pyruvate oxidation, inhibited O_2-uptake and also

TABLE I

EFFECT OF ARSENITE (0.01 M) ON RELEASE OF C-1 AND C-6 OF GLUCOSE AS CO_2

EXPT.	TIME	CONTROL			+ ARSENITE		
		% OF C-1 CONVERTED TO CO_2	% OF C-6 CONVERTED TO CO_2	C_6/C_1	% OF C-1 CONVERTED TO CO_2	% OF C-6 CONVERTED TO CO_2	C_6/C_1
A	0–3 hr	3.5	3.9	*1.10*	3.4	2.1	*0.62*
	3–6 hr	1.9	1.8	*0.95*	1.7	1.3	*0.76*
B	0–2½ hr	1.8	2.2	*1.22*	1.3	0.7	*0.54*
	2½–5 hr	1.5	2.2	*1.45*	1.1	0.6	*0.55*

brought about aerobic fermentation in this tissue as it does in corn roots (6). At a concentration of 0.01 M the O_2 uptake was some 44 % of the control rate and the RQ was 1.7. This was the concentration of arsenite used in the two experiments described in table I. First, it should be pointed out that the high ratios in the controls are due to the fact that they were perforce determined in glucose-free phosphate after a short pretreatment with glucose. Thus only a small amount of glucose was absorbed and its metabolism had already proceeded to a stage where the rate of release of $C\text{-}6_1$ relative to that of $C\text{-}1_1$ was higher than it is when measurements are made immediately after adding glucose. The effect of the arsenite, clearly, is drastically to reduce the C_6/C_1 ratios. This was brought about principally by an inhibition of the release of C-6 as CO_2; the release of C-1 was inhibited to a much smaller degree.

II. Experiments with DNP. A concentration of 0.000025 M DNP doubled the O_2-uptake of tissue which had been pretreated with glucose for 4 hours. No change was induced in the RQ. Although the primary effect of DNP is to uncouple phosphorylation from electron transfer, it is clear that this leads to marked stimulations in glycolysis in plant tissues (4). It was therefore held that DNP might increase the proportion of glucose catabolized via the EMP pathway. The data in table II show that the CO_2 output from both carbons 1 and 6 was stimulated by DNP. But whereas the output of C-1 was increased by a relatively small amount, that of C-6 was more than doubled in the first periods of measurement. The result was a significant increase in the C_6/C_1 ratios. Shaw and Samborski (16) report an increased C_6/C_1 ratio in wheat leaves after DNP treatment. However in their experiments this could not be ascribed to a stimulation of the release of C-6 as CO_2.

III. Experiments with methylene blue. Stimulation of carrot respiration by this reagent has been demonstrated frequently (12, 17). The ability of methylene blue to accept electrons from reduced triphosphopyridine nucleotide suggested that this agent might stimulate the participation of the PP pathway in the respiration. At a concentration of 0.001 M methylene blue increased the O_2-uptake of carrot slices by 30 to 40 % and the RQ was unchanged. The effects on the release of C-1 and C-6 of glucose are shown in table III. As expected, the output of $C^{14}O_2$ was stimulated by the methylene blue. But there was a clear distinction between the effects on the individual carbons; the stimulation of release of C-1 was much more marked than that of C-6 and the C_6/C_1 ratios were consequently lowered.

IV. Experiments with iodoacetate. Turner's work with carrots showed that at certain levels of iodoacetate there was some differentiation between effects on respiration and fermentation (20). Although the explanation of this is far from clear (13), it seemed possible that the greater apparent resistance of aerobic respiration might be due to participation of the PP pathway. It was first determined that under our conditions, 0.00006 M iodoacetate was the most promising differentiating concentration. The iodoacetate gradually inhibited fermentation: the rate

TABLE II

EFFECT OF DINITROPHENOL (0.000025 M) ON RELEASE OF C-1 AND C-6 OF GLUCOSE AS CO_2

EXPT.	TIME	CONTROL			+ DNP		
		% OF C-1 CONVERTED TO CO_2	% OF C-6 CONVERTED TO CO_2	C_6/C_1	% OF C-1 CONVERTED TO CO_2	% OF C-6 CONVERTED TO CO_2	C_6/C_1
A	0–3 hr	0.39	0.39	*1.00*	0.66	0.86	*1.30*
B	0–2 hr	1.37	0.82	*0.60*	1.53	1.69	*1.10*
	2–4 hr	0.42	0.55	*1.31*	0.74	0.95	*1.29*
C	0–2 hr	0.51	0.34	*0.67*	0.68	0.72	*1.06*
	2–4 hr	0.27	0.25	*0.93*	0.28	0.31	*1.11*

TABLE III

EFFECT OF METHYLENE BLUE (0.001 M) ON RELEASE OF C-1 AND C-6 OF GLUCOSE

EXPT.	TIME	CONTROL			+ METHYLENE BLUE		
		% OF C-1 CONVERTED TO CO_2	% OF C-6 CONVERTED TO CO_2	C_6/C_1	% OF C-1 CONVERTED TO CO_2	% OF C-6 CONVERTED TO CO_2	C_6/C_1
A	0–3 hr	1.5	0.8	*0.53*	3.8	1.1	*0.29*
B	0–2 hr	1.5	0.7	*0.47*	2.4	0.9	*0.37*
	2–4 hr	5.5	2.0	*0.36*	9.9	2.8	*0.28*
C	0–2 hr	1.5	0.7	*0.47*	2.5	1.1	*0.44*

was halved after 3 hours. The inhibitor did not reduce the rate of O_2 uptake below the initial value but it did prevent the development of the respiratory rise shown by the controls. The effects on the release of C-1 and C-6 were studied with disappointing results. In all of the experiments production of CO_2 from both C-1 and C-6 was inhibited. In some experiments the release of C-6 was more severely inhibited than that from C-1 but in others the converse occurred. Neither result could be obtained consistently.

RELEASE OF $C^{14}O_2$ FROM SPECIFICALLY LABELED SUBSTRATES. *I. Pyruvate.* Since pyruvate is a key intermediate in the breakdown sequences, it was of value to know the fates of its individual carbons. It was first established that, even at double the concentration used in these experiments, pyruvate had no detectable effect upon either the O_2-uptake or the RQ of freshly cut carrot slices.

The release of $C^{14}O_2$ from specifically labeled pyruvates is shown in figure 3 of the succeeding paper (1). The rapid and complete recovery of C^{14} from pyruvate-1-C^{14} indicated that all of the added pyruvate was promptly metabolized. However, the curve for release of $C^{14}O_2$ from pyruvate-2-C^{14} showed a noticeable lag, its slope never exceeded half that from pyruvate-1-C^{14} and the release slowed to a low rate after 12 hours. From pyruvate-3-C^{14} the release of $C^{14}O_2$ was barely detectable until 6 hours and the rate and final yield were considerably lower than from pyruvate-2-C^{14}. Although release of C-1 as CO_2 was complete in 12 hours some 40 % of C-2 and 56 % of C-3 were retained in the tissue even after 24 hours. These results are consistent with a complete conversion of pyruvate to acetyl CoA, and the sequential but incomplete release of carbons 2 and 3 of the pyruvate as the acetyl CoA was metabolized through the TCA cycle. The considerable retention of carbons 2 and 3 of pyruvate (equivalent to carbons 1, 2, 5, and 6 if breakdown were exclusively via the EMP sequence) is ascribed to a diversion of TCA intermediates and hence an incomplete dissimilation of the acetyl CoA. (see Neal & Beevers 15).

II. Glucose. The progress of $C^{14}O_2$ production from specifically and uniformly labeled glucose is shown in figure 1. Of the total carbon added as glucose 27.3 % had appeared as CO_2 at the end of the experiment, but this was contributed to in very different measure by the individual carbons in the molecule. The percentage of the individual carbons which had appeared in the respired CO_2 were as follows: C-1 28.8 %; C-2 21 %; C_3+C_4 83.6 %; C-6 15.7 %. If the contribution of C-5 had been the same as that of C-2, the predicted value for the average recovery would have been 27.9 %; the observed value was 27.3 %. The agreement constitutes an overall check on the validity of the individual values.

The contributions of the last five carbons of the glucose (i.e., ($C_{3,4} > C_2 = C_5 >$ C-6) are precisely those predicted if the glucose unit had been cleaved by the EMP sequence and the pyruvate metabolized as shown in figure 1.

The contribution of C-1 is not understandable on this basis. In the first 9 hours C-1 was being given off at a rate almost twice that of C_2 and three times

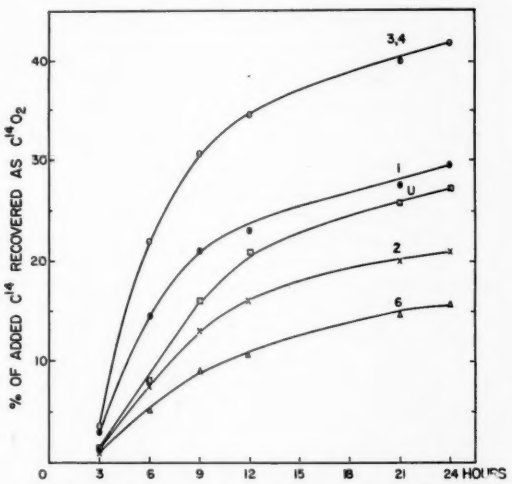

FIG. 1. Time course of $C^{14}O_2$ output from differently labeled glucoses. Curves 1, 2, 3, 4, 6, and U (Uniform) refer to the position of the C^{14} in the respective substrates. 1.3 g carrot slices received 2 μmoles of the appropriate glucose.

TABLE IV

INCORPORATION OF C-1 AND C-6 OF GLUCOSE INTO MAJOR FRACTIONS

SUBSTRATE	GLUCOSE-1-C^{14} % ABSORBED C^{14}	GLUCOSE-6-C^{14} IN FRACTIONS [1]
CO_2	22	8
Alcohol soluble	58	67
Alcohol insoluble	20	24
Total recovered	100	99

[1] Average values from three experiments.

that of C_6. Clearly this is the kind of result expected if part of the glucose were being channeled through the PP pathway.

The early appearance in the respired CO_2 of the first carbon of the glucose was not followed by a period in which C-6 showed a corresponding predominance. At no time in the experiment did the rate of release of $C^{14}O_2$ from glucose-6-C^{14} exceed that from glucose-1-C^{14}. If glucose is oxidized completely, by whatever pathway, the total contribution of C-1 equals that of C-6 (*2*). In this tissue, even after 23 hours no such equivalence was found. If the early release of $C^{14}O_2$ from C-1 of the glucose is attributed to the initial decarboxylation step in the PP pathway, it is clear that not all of the carbons in the resulting pentose unit were released by continued recycling in this sequence.

FATE OF 5-CARBON UNIT. It was clearly very important to discover whether the carbon not appearing as CO_2 was retained as a pentose or an immediate derivative or whether it was more extensively metabolized. This question was answered by comparing the distribution of radioactivity in various components of samples of tissue which had been supplied with either glucose-1-C^{14} or glucose-6-C^{14}. The tissues were fractionated 7 hours after adding the labeled glucose. By this time more than 90 % of the added glucose had been absorbed and the excess of C-1 (21 %) over C-6 (6 %) in the respired CO_2 was near the maximum. The distribution of C^{14} in the major fractions separated from the tissue is shown in table IV. It should be noted that although the relative contributions of C-1 and C-6 to CO_2 differed markedly, this difference (15 %) was only a relatively small fraction of the added C^{14}. Thus virtually complete recovery of the residual C^{14} in the various fractions was necessary before any reliable evidence could be obtained of the fate of the pentose formed in the PP pathway. The data in tables V and VI meet this requirement. Furthermore, the total C^{14} in the fractions separated from the tissue supplied with glucose-6-C^{14} exceeded that from glucose-1-C^{14} by virtually the same figure as that by which the C^{14} in the CO_2 from glucose-1-C^{14} exceeded that from glucose-6-C^{14}. In each of the experiments shown in table IV this difference is accounted for mainly by the alcohol soluble fraction.

Table V shows the distribution of radioactivity after complete fractionation of the tissue. Three aspects of these data are stressed:

I. Even after hydrolysis of the alcohol insoluble fraction 96 to 97 % of the absorbed activity was finally recovered in the different fractions.

II. The extra activity retained in the tissue which had been supplied with glucose-6-C^{14} was not confined to one fraction.

III. In relation to the distribution of activity from glucose-1-C^{14}, glucose-6-C^{14} made much greater contributions to the organic acid, amino acid, and protein fractions.

Each of these fractions was separated into its main components, and attempts were made to determine whether or not the activity derived from glucose-6-C^{14} was distributed any differently from that derived from glucose-1-C^{14}. Chromatograms of the neutral fraction of the aqueous extract revealed areas which corresponded to sucrose, glucose, fructose, and raffinose. No pentose was detected. The data in table VI show that the bulk of the activity was present in sucrose

TABLE V

DETAILED ANALYSIS OF FRESHLY CUT CARROT TISSUE AFTER SUPPLYING GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} FOR 7 HOURS

FRACTION	GLUCOSE-1-C^{14}		GLUCOSE-6-C^{14}	
	CPM	% OF UPTAKE	CPM	% OF UPTAKE
CO_2	5,201	21	2,088	6
Soluble carbohydrates	10,760	44	17,820	48
Organic acids	1,223	5	3,641	10
Amino acids	2,350	10	5,285	14
Proteins	1,195	5	2,650	7
Remainders of hydrolysate	1,940	8	2,740	7
Hexane extract of soluble fraction	800	3	1,750	5
Totals	23,469	96	35,974	97

TABLE VI

CHROMATOGRAPHIC SEPARATION OF COMPONENTS OF SUGAR AND ACID FRACTIONS ISOLATED FROM TISSUE SUPPLIED WITH GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14}

SUBSTRATE	GLUCOSE-1-C^{14} % OF C^{14} ELUTED	GLUCOSE-6-C^{14} % OF C^{14} ELUTED
Sugar Fraction		
Raffinose ?	3	2
Sucrose	80	79
Glucose	9	11
Fructose	8	8
Acid Fraction		
Malic	73	78
Citric + isocitric	21	16
Succinic	6	6

and that the percentage distribution of the activity from one and six labeled glucose was identical. The areas of the chromatograms which corresponded with the position of known ribose, xylose, and arabinose were eluted. No radioactivity was detected in these eluates. Radioautograms, exposed for as long as 4 months, confirmed that neither extract contained any appreciable amounts of radioactive pentose and that the bulk of the activity was confined to sucrose. Analysis of the residual fractions of the hydrolysate revealed slight activity in hexose: no radioactive pentose was found in either extract.

The data in table VI show that although glucose-6-C^{14} contributed twice as much of its activity to the organic acid fraction as did glucose-1-C^{14}, the distribution of the activity was the same in both fractions.

Chromatograms of the free amino acids, and the amino acids derived from the alcohol insoluble fractions revealed a range of amino acids comparable to that obtained by Stewards group (18). The activity present in these fractions was so low that, even after 5 months, radioautography gave no clue to the distribution of activity within each fraction. No differences between fractions derived from tissues which had been fed -1- or -6- labeled glucose were found.

DISCUSSION

These results indicate the main routes of glucose dissimilation in freshly cut carrot tissue and contribute to our knowledge of the relationship between the EMP and PP pathways.

The previous evidence for operating the EMP and PP pathways has been considerably augmented. Arsenite, DNP, and methylene blue all had pronounced effects upon the relative contributions carbons 1 and 6 of glucose to the respired CO_2. These changes strongly implicate both pathways in the catabolism of glucose and are entirely consistent with the predicted effect of each treatment upon the balance between the two pathways. The contribution of the EMP pathway was increased by treatment with DNP. These results indicate that, as anticipated from our earlier work (4), treatment with DNP led to a stimulation of glycolysis. The proportion of glucose oxidized via the PP pathway was increased both by the restriction of the EMP pathway (pyruvate oxidation) by arsenite and by the stimulation of the PP pathway by methylene blue. Cahill et al (8) have reported similar effects of DNP and methylene blue upon the C_6/C_1 ratio of slices of rat liver.

It is difficult to explain the effects of iodoacetate upon the C_6/C_1 ratio. Even at pH 5.0 iodoacetate is known to enter plant tissues slowly. The variation in our results may be connected with this slow entry and the fact that substantial inhibition of fermentation did not develop until the oxygen uptake of the untreated control had increased markedly. It is also possible that iodoacetate inhibited some enzyme other than triose phosphate dehydrogenase.

Good evidence for the operation of EMP and PP pathways is afforded by the rates of release of $C^{14}O_2$ from specifically labeled substrates. The low C_6/C_1 ratio, due to the large and early contribution of the first carbon of the glucose molecule to the respired CO_2, is characteristic of the PP pathway. It may be argued that the early release of activity from glucose-1-C^{14} could also have resulted from the catabolism of glucose via the Entner-Doudoroff pathway. This possibility is ruled out by the fact that the release of $C^{14}O_2$ from glucose-3, 4-C^{14} was greater and more rapid than that from glucose-1-C^{14}. The close correspondence between the release of activity from 3-4, 2, and 6 labeled glucose with that from 1, 2, and 3 labeled pyruvate, respectively, indicates that much of the glucose was converted to pyruvate and that the labeling of this pyruvate was consistent with its formation via the EMP pathway. When the previous evidence for the two pathways is considered with that presented in this paper there remains little doubt that both make substantial contributions to the oxidation of glucose.

Perhaps the most important question arising from this conclusion is the fate of the pentose produced in the PP pathway and the relevance of this knowledge to any assessment of the quantitative importance of each pathway. We emphasize here the failure of C-6 to appear in CO_2 to the same extent as C-1 even after long intervals. In the experiment shown in figure 2 only 27 % of the absorbed glucose carbon was converted to CO_2. Yet, from the contributions of carbons 1 and 6 to the CO_2 it is clear that at least 15 % of the absorbed glucose was converted to pentose. Even in 24 hours little of C-5 this pentose was apparently oxidized to CO_2. But there was no evidence at all that tissues which had been fed glucose-6-C^{14} contained radioactive pentose or its immediate derivatives in amounts sufficient to account for that known to have been formed by the PP pathway. The analytical data prove in fact that the pentose was not retained in the tissue as such but was extensively metabolized. In an experiment

in which 96 % of the added C^{14} was accounted for it was found that glucose-6-C^{14} did not contribute C^{14} to any compound which was not also labeled by glucose-1-C^{14}. The extra C^{14} in the tissue metabolizing glucose-6-C^{14} was distributed among the organic acid, amino acid, protein, and sugar fractions. It was also shown that the distribution of C^{14} in the components of the sugar fraction and in those of the organic acid fraction was the same whether the C^{14} was derived from 1 or 6 labeled glucose. The contribution of C^{14} from glucose-1-C^{14} to the organic acid, and protein fractions are consistent with the oxidation of glucose to pyruvate via the EMP pathway.

It is proposed that pentose units produced in the PP pathway gave rise to triose and thence pyruvate and that this pyruvate was subsequently metabolized together with that produced solely via the EMP pathway. The evidence for this hypothesis is threefold. First, the C^{14} representing the pentose formed from glucose-6-C^{14} was almost entirely accounted for in compounds which are derived from pyruvate. Second, the striking similarity in the relative distribution of activity from 1 and 6 labeled glucose indicates that the products of both pathways were eventually metabolized together. Third, the production of $C^{14}O_2$ from specifically labeled glucose and pyruvate indicates that, apart from the release of C-1 of the glucose in the PP pathway, the majority of the glucose was first converted to pyruvate before any CO_2 was formed. This hypothesis is entirely consistent with our knowledge of the two pathways. Pentose formed in the PP pathway may be converted to hexose and triose by the action of transketolase and transaldolase. In a tissue in which glucose is being oxidized via both pathways it is probable that this triose would be converted to pyruvate rather than condensed to form hexose. Under these conditions, much of the hexose produced by the PP pathway would be converted to pyruvate either via the EMP pathway or by recycling through the PP pathway. Unless this recycling were extensive most of the pyruvate formed from the pentose would be derived from carbons 4, 5, and 6 of the original glucose molecule and would thus *be indistinguishable from that formed* solely via the EMP pathway.

There are good indications of the routes by which this pyruvate was subsequently metabolized. Three types of evidence imply that pyruvate was metabolized via the TCA cycle. First, arsenite, which is known to inhibit the oxidation of keto acids, caused marked aerobic fermentation. Second, appreciable amounts of C^{14} from glucose-C^{14} were detected in succinate, malate, citrate, and isocitrate. Third, the pattern of the release of $C^{14}O_2$ from specifically labeled pyruvate indicates its oxidation by the TCA cycle. The retention in the tissue of much of the C^{14} added as 2 and 3 labeled pyruvate and the labeling of amino acids and proteins by 1 and 6 labeled glucose is no doubt due to the fact that the two-carbon units derived from pyruvate served as a source of carbon for the synthesis of amino acids and proteins. Those glucose molecules which were metabolized exclusively via the EMP sequence would have contributed equal proportions of C-1 and C-6 to compounds derived from pyruvate. The excess of C-6 in such components, its consequent non-appearance as CO_2, are ascribed to that fraction of the glucose metabolized by the PP sequence. The conversion of C-6 of the glucose to C-3 of pyruvate by this pathway and the retention of a large fraction of this in cellular constituents would lead to the observed results.

Fractionation of tissues which had been fed -1- or -6- labeled glucose revealed that at least a third of the absorbed glucose was converted to sucrose. Not only did a similar percentage of both isotopes appear in the sugar fractions but the distribution of this activity among the various components was identical in each instance. Thus it appears that the main routes of metabolism of supplied glucose in freshly cut carrot tissue are conversion to sucrose, oxidation via the EMP pathway, and oxidation via the PP pathway. There is good evidence that the two pathways of glucose oxidation are closely integrated and that in this tissue they cannot be pictured as two entirely separate enzyme systems. This close relationship makes it difficult to determine the exact proportion of glucose oxidized through each pathway.

In general, these results emphasize the need for caution in calculating the proportion of glucose oxidized through each pathway. Such calculations must now take into account the facts that the pentose formed in the PP pathway may be metabolized to pyruvate and that there may be a differential and indefinite retention of the carbons of the acetyl units formed from this pyruvate and that produced by the EMP sequence. It does not now seem possible to base these calculations solely upon the rate of release of $C^{14}O_2$ from specifically labeled glucose.

The C_6/C_1 ratio shows the minimum contribution of the PP pathway to the release of C-1 of glucose and a low ratio is of course a good qualitative indication of its participation in respiration. Calculation of the contributions to total glucose oxidation based upon this ratio only are inaccurate. The results of the Oregon group (3, 10, 22) and the present work show that certain of the glucose carbons (particularly 6, 2, 5, and 1) may be drained off into synthetic processes even in non-growing tissues. Thus estimates of the glucose catabolized which are based on the release of C-1 and C-6 would be too low. The error would be larger where extensive drainage of two carbon units from the TCA cycle was occurring.

This difficulty was overcome to some extent by the Oregon group (3, 10, 22) who calculated the amount of glucose involved in catabolic reactions from the yields of $C^{14}O_2$ from glucose -3,4-C^{14} as well as that from glucose-1-C^{14} and glucose-6-C^{14}. These authors estimated the participation of the two pathways from the radiochemical yields from four specifically labeled glucoses. Certainly the use of other labeled glucose samples allows a much more complete picture to be drawn and leads to somewhat

more accurate estimates of the contributions to glucose catabolized (as opposed to glucose converted to CO_2). Although this method has been used frequently, it does not necessarily give a reliable answer.

Some of the assumptions on which the calculation is necessarily based are open to question, and particularly so since they seem on balance to make for an underestimate of the importance of the PP pathway. On the basis of the present work we would question the assumption that pentose formed from the PP pathway is not promptly catabolized. In view of the known spreading of C-6 to C-1 of glucose in plant cells (11), it is also probable that the early reactions of the PP pathway contribute significantly to the appearance of C-6 as CO_2. It should also be emphasized that the triose phosphate generated in the PP pathway arises predominantly from carbons 4, 5, and 6 of the original glucose and will therefore give rise to pyruvate whose carboxyl group corresponds to C-4 of glucose. The C^{14} in the CO_2 from glucose-3,4-C^{14} cannot therefore be ascribed solely to decarboxylation of pyruvate produced by the EMP sequence. Finally, no account is taken of the complication arising from retention of the carbon of the acetate unit.

Other methods of calculation, such as those of Shaw and Samborski (16) must be treated with similar reservations. Yet another proposed calculation (9) depends on adding arsenite to induce pyruvate accumulation. Apart from the fact that pyruvate accumulating in plant tissues is promptly converted to acetaldehyde and alcohol, this method is inapplicable because of the effects of arsenite on the release of C^{14} from specifically labeled glucose described in this paper.

In respect to our own work, it seems that in addition to the information we have already we require the following values before more meaningful estimates can be made.

I. The proportion of pyruvate derived from pentose units.

II. The extent to which recycling in the PP pathway contributed to the yield of $C^{14}O_2$ from 2, 3, 4, and 6 labeled glucose.

III. The extent to which glucose produced in the PP pathway was converted to sucrose.

Summary

Experiments on the respiration of freshly cut carrot slices have demonstrated the participation and interrelations between the two major pathways of glucose breakdown, the EMP sequence, and the PP sequence. The following points are emphasized:

I. The prevailing balance between the proportions of glucose molecules which initially enter the two sequences can be altered by suitable treatments. These alterations are understandable on the selective action of the agents employed. Thus DNP increased the amount of glucose metabolized by the EMP sequence, arsenite decreased this value, and methylene blue increased the participation of the PP pathway.

II. The slices utilize added pyruvate actively, C-1 appears quantitatively in the CO_2 while C-2 and C-3 appear later and in sequence. About 40 % of C-2 and 55 % of C-3 were retained in metabolic products after 24 hours.

III. The time curves for the release of $C^{14}O_2$ from specifically labeled glucose samples show that very different contributions are made by the individual carbons, with a corresponding differential retention in metabolic products. Thus carbon from positions 1, 3, and 4 makes up the bulk of the respired CO_2 with smaller amounts from C-2 and C-5 and a yet smaller contribution from C-6. It appears that some pyruvate is produced directly from the EMP sequence and its carbons suffer the fate predicted in II.

IV. Detailed analysis of the tissue in which all of the added C^{14} was accounted for showed that the 5-carbon unit corresponding to the excess of C-1 released as CO_2 did not remain as a pentose or an immediate derivative. C-6 of the glucose was not found in any compound which was not also labeled by C-1, but it did make a bigger contribution to the organic acid, amino acid, and protein components.

V. It is concluded that the 5-carbon unit is further metabolized in the PP sequence to give rise in part to triosephosphate and so to pyruvate. The result of superimposing this on the exclusive breakdown by the EMP is a mixed pyruvate pool with C-6 and C-1 primarily in the methyl group but richer in C-6 than C-1. The metabolism of this pyruvate by the pattern established in II would go far to account for the analytical results. The bearing of these results on the problem of calculating the participation of the two pathways is discussed.

Acknowledgment

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PENTOSE PHOSPHATE PATHWAY AS A MAJOR COMPONENT OF INDUCED RESPIRATION OF CARROT AND POTATO SLICES [1, 2]

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When washed at moderate temperatures, slices of many underground storage organs show a steady rise in their rate of respiration until it reaches a value two to five times that of freshly cut tissue. Laties (3) has recently reviewed our knowledge of these changes in respiration. In accordance with his suggestion, the respiration which develops gradually on washing will be referred to as induced respiration. It is important to distinguish between this induced respiration and any immediate increase in respiration caused by cutting the whole organ. Stiles and Dent (9) and Turner and Hanly (10) have reported the development of such an induced respiration in washed slices of carrot tissue. In a previous paper (1) we have shown that in freshly cut carrot tissue glucose is oxidized through both the Embden-Meyerhof-Parnas (EMP) glycolytic pathway and the pentose phosphate (PP) pathway, and that it is possible to alter the relative contributions of these two pathways to glucose oxidation. On indirect evidence, Laties (4) has suggested that the development of induced respiration involves qualitative as well as quantitative changes in the respiration. In view of our knowledge of the respiratory pathways in freshly cut carrot tissue it was worthwhile to examine the pathways of glucose oxidation in washed tissue. This paper describes some further characteristics of the basal and induced respiration in carrot tissues and presents evidence that the development of the induced respiration was accompanied by an increase in the proportion of glucose oxidized through the PP pathway. Evidence is also presented that a similar increase in the activity of the PP pathway accompanied the development of the induced respiration of slices of potato tubers.

MATERIALS AND METHODS

Different varieties of carrots and potatoes were used. Slices of the carrot roots and the potato tubers were cut and sampled as described earlier (1). Comparisons between fresh and washed tissue were made only within the same batch of replicate samples. Half the samples were used at once and were designated freshly cut tissue. The remaining samples were placed in the washing apparatus and referred to as washed tissue.

The discs were washed in an apparatus modelled on that used in the study of beech mycorrhizas by Harley and McCready (2). Each sample was washed separately in 80 to 100 ml of 0.02 M potassium phosphate at pH 5.0. The discs were washed for 24 hours at room temperature. The buffer was changed three times during this period. The discs were agitated continuously by bubbling sterile air through the washing solution. The complete apparatus and the buffer were sterilized before use. We found no evidence of microbial contamination of the washed samples.

For respiratory measurements 650 mg fresh weight of tissue were placed in 2.0 ml of 0.03 M potassium phosphate at pH 5.0. C^{14} labeled glucose and pyruvate were supplied to 1.3 g fresh weight of tissue in 4.0 ml buffer. The initial concentration of the glucose and the pyruvate was 0.5 μ moles/ml. The techniques used in the feeding experiments, in the measurement of radioactivity, the collection of $C^{14}O_2$, and the fractionation of the tissue have already been described (1).

RESULTS

DEVELOPMENT AND CHARACTERISTICS OF INDUCED RESPIRATION: Figure 1A (control) shows the gradually increasing O_2 uptake characteristic of storage tissue. From an initial value of 67 microliters/hour, the rate of O_2 uptake of the carrot slices rose to more than 150 μ liters/hour in the 10 hours during which the tissue was being shaken in Warburg respirometers. The control curve in figure 1B established that a similar rise had occurred in slices which had been aerated in the washing apparatus for 24 hours. During the subsequent 3 hours the washed slices showed a further small rise in respiratory rate and the rate was maintained at a level more than twice that of the original samples for several hours.

Respiratory Quotient and effects of added substrates: At no time did the RQ vary significantly from unity, and adding glucose and pyruvate in the amounts used in later experiments did not induce significant changes in O_2 uptake or in CO_2 output of the slices.

Effects of methylene blue, 2,4-dinitrophenol (DNP) and inhibitors: Adding 0.000025 M DNP to freshly cut tissues led to an immediate and maintained stimulation of respiration. The new rate was close to that achieved by the controls 10 hours later, i.e., after the full development of the induced respiration. On

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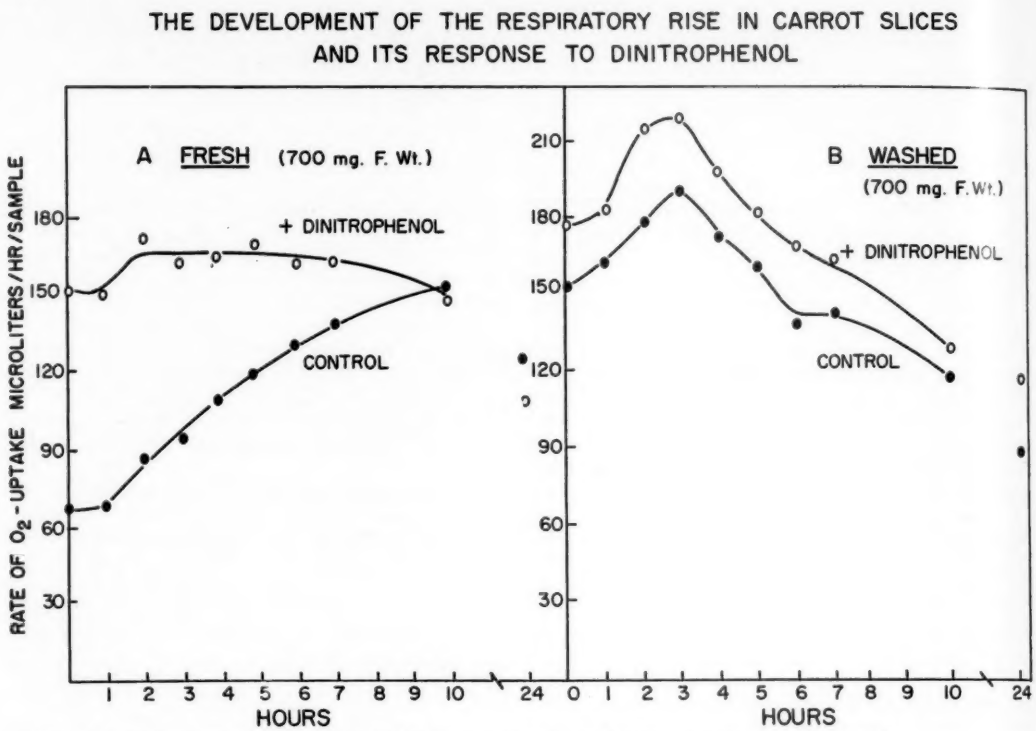
THE DEVELOPMENT OF THE RESPIRATORY RISE IN CARROT SLICES AND ITS RESPONSE TO DINITROPHENOL
A FRESH (700 mg. F. Wt.)
+ DINITROPHENOL
CONTROL
B WASHED (700 mg. F. Wt.)
+ DINITROPHENOL
CONTROL
RATE OF O2 - UPTAKE MICROLITERS/HR/SAMPLE
HOURS
HOURS

FIG. 1. Development of induced respiration in carrot slices and its response to 2,4-dinitrophenol.

the contrary, adding DNP to washed slices (fig 1B) stimulated the respiration by less than 20 %.

Adding arsenite (table I) inhibited the O_2 uptake of both fresh and washed slices; the latter were somewhat more sensitive to the inhibitor. Arsenite had the further property, not shared by methylene blue, of completely preventing the development of the respiratory rise even at concentrations (0.001 M) which had relatively small effects on the initial rate of O_2 uptake. It will be recalled (1) that iodoacetate also prevents development of the induced respiration.

Over a wide range of concentrations, methylene blue stimulates the respiration of both washed and fresh tissue to equal degrees (table II).

Uptake of glucose by fresh and washed slices: After development of the induced respiration, glucose is absorbed more rapidly by the carrot slices (table III). A similar effect is noted for potato discs.

Stimulation of fermentation during washing: It was of some interest to find that the fermentation rates

TABLE I

EFFECTS OF ARSENITE ON O_2-UPTAKE OF FRESH AND WASHED CARROT SLICES

CONC OF ARSENITE (M)	0.02	0.01	0.004	0.002	0.001
	O_2 UPTAKE AS % OF CONTROL RATE				
Fresh tissue	37	54	52	70	85
Washed tissue	37	44	48	43	52

TABLE II

EFFECTS OF METHYLENE BLUE ON O_2 UPTAKE OF FRESH AND WASHED CARROT SLICES

CONC OF METHYLENE BLUE (M)	0.002	0.001	0.0002	0.0001
	O_2 UPTAKE AS % OF CONTROL RATE			
Fresh tissue	161	160	152	128
Washed tissue	154	140	132	140

TABLE III

EFFECT OF WASHING ON ABSORPTION OF GLUCOSE BY CARROT AND POTATO DISCS

TIME (HR)	% ADDED GLUCOSE ABSORBED			
	CARROT DISCS		POTATO DISCS	
	FRESH	WASHED	FRESH	WASHED
3	17	75	17	35
6	60	89	47	66
9	93	90	82	60

TABLE IV

EFFECTS OF WASHING ON RATES OF AEROBIC RESPIRATION AND OF FERMENTATION IN NITROGEN (CARROT SLICES)

	FERMENTATION			RESPIRATION		
	FRESH	WASHED	% STIMULATION	FRESH	WASHED	% STIMULATION
A	41	44	7	39	92	136
B	61	97	59	32	99	210

The values for fermentation are the averages of four determinations of CO_2 output/hour/750 mg tissue. Those for respiration are the averages of three determinations of O_2 uptake/750 mg tissue. A and B are separate experiments.

of washed slices, though greater than those of fresh tissue were stimulated to a much smaller degree than were the corresponding rates of O_2 uptake (table IV).

Changes in C_6/C_1 ratio during development of respiratory rise: The first indication that a change in metabolism occurred during washing was an unexpected fall in the C_6/C_1 ratio a few hours after adding the glucose to freshly cut tissue. The usual (and expected) finding is that the contribution of C-6 relative to that of C-1 increases steadily with time and the initial ratio is that aimed for in looking for the participation of the PP pathway. In all our experiments with fresh carrot slices of varied origin, the ratio during the first 2 to 3 hours was remarkably constant at about 0.48. However in every experiment the ratio observed during the succeeding 4-hour period [i.e., at a time coinciding with the beginning of the respiratory rise (fig 1A)] was significantly lower than this. This marked change in C_6/C_1 ratio was due to the relatively greater increase in the contribution of C-1 to CO_2. Table V shows the results of five experiments in which these changes were measured with time. It should be recognized that the fall in the ratio occurred despite the fact that the release of C-6 as CO_2 was increasing during this time, and that a subsequent increase in the ratio would occur when the release of C-1 neared completion. These findings suggested that development of the induced respiration was accompanied by an increase in amount of glucose channeled through the PP pathway.

When slices which had been washed for 24 hours were examined, it was gratifying to find that the C_6/C_1 ratio was very low—i.e., about half that observed in the fresh material for the corresponding period (table VI). A significant finding for the hypothesis that the PP pathway was an important component of the induced respiration was that, in contrast to the situation in fresh tissue, there was no temporary decline in the ratio during the subsequent periods (table VI). Instead, it rose steadily as would be expected if the release of C-6 depended to some extent on reactions subsequent to the release of C-1, and no further change had occurred in the balance between participating pathways.

TABLE VI

C_6/C_1 RATIOS IN WASHED CARROT SLICES

TIME (HR)	RATIO
0–3	0.21
3–6	0.39
6–9	0.65
9–12	0.70
12–24	1.0

EXPERIMENTS WITH DIFFERENTLY LABELED GLUCOSE: The order of release of the individual carbons from glucose by fresh slices was established earlier (1). Similar observations on washed slices revealed the important changes shown in figure 2. The first point to be noticed is that glucose is metabolized more rapidly by the washed slices, due at least in part to the fact that the uptake of glucose is more rapid. A more important point however, is the contrasting behavior

TABLE V

DECLINE IN C_6/C_1 RATIO OF FRESHLY CUT CARROTS DURING DEVELOPMENT OF INDUCED RESPIRATION

A		B		C		D		E	
TIME (HR)	RATIO	TIME (HR)	RATIO	TIME (HR)	RATIO	TIME (HR)	RATIO	TIME (HR)	RATIO
0–2½	0.42	0–3	0.49	0–3	0.49	0–2	0.48	0–3	0.50
2½–5¼	0.25	3–6¼	0.34	3–6	0.35	2–4	0.36	3–6	0.33
5¼–8	0.35	6¼–9½	0.53	6–9	0.40	4–6	0.24		
8–11½	0.68								

A, B, C, D, E are separate experiments.

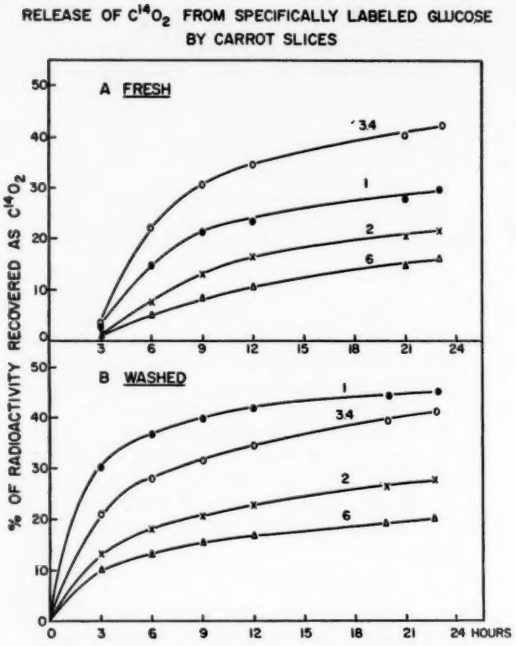

FIG. 2. Release of $C^{14}O_2$ from specifically labeled glucose samples by fresh and washed carrot slices. Numbers on the curves refer to the position of C^{14} in the glucose provided.

of C-1 in relation to that of the other carbons. Washing leads to a tremendous increase in the yield of CO_2 from C-1. This change contrasts strikingly with the fact that the yields from C-2,3,4, and 6 are barely altered. Not only is the production of CO_2 from C-1 more than three times greater than that of C-6 in the washed slices; it is now half as much again as that from C-3,4. This result which has been obtained in each of our experiments is regarded as a highly significant one and crucial for the hypothesis in hand. Glucose-U-C^{14} was also included in the experiment with washed slices in figure 2; 33.4 % of its carbon had been recovered as CO_2 at the end of the experiment. We can calculate that, as in fresh slices, the contribution of C-5 must have been close to that from C-2. From the values in figure 2, we can calculate the contribution of each carbon of the glucose to the CO_2 at any time. The values for 3 hours and 23 hours are shown in table VII. Of the carbon in the CO_2 at the end of 3 hours some 27 % had arisen from C-1 of the glucose while even after 23 hours the contribution was more than 22 %. In each of these periods, less than 10 % of C-6 had appeared as CO_2.

EXPERIMENTS WITH PYRUVATE: The earlier experiments established that fresh tissue is not characterized by any inability to carry out reactions of the TCA cycle. On the contrary, the respiration was inhibited by arsenite and released the various carbons of pyruvate as CO_2 in the predicted manner. Figure 3 compares the abilities of fresh and washed tissues to metabolize added pyruvate. The two sets of curves are clearly similar. The washed slices deal with the pyruvate somewhat more rapidly but the final yields as CO_2 from the individual carbons are remarkably close.

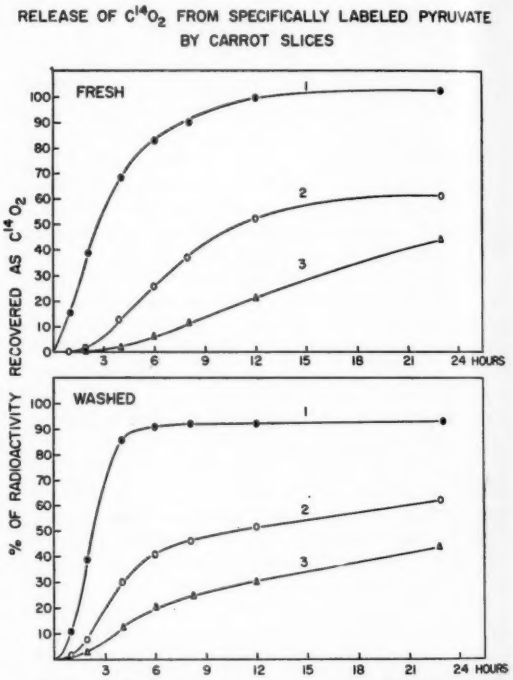

FIG. 3. Release of $C^{14}O_2$ from specifically labeled pyruvate samples by fresh and washed carrot slices. Numbers on the curves refer to the position of C^{14} in the pyruvate provided.

TABLE VII

% CONTRIBUTIONS OF INDIVIDUAL CARBONS TO RESPIRATORY CO_2 FROM WASHED CARROT SLICES

TIME (HR)	C-1	C-2	C-3	C-4	C-5(CALC)	C_6	U.L.*
3	27.6	12.0	19.6	19.6	12.0	9.1	14
23	22.6	13.5	20.1	20.1	13.5	9.9	33

* % Added C^{14} recovered from glucose-U-C^{14}. (= % added glucose carbon converted to CO_2).

TABLE VIII

UTILIZATION OF GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} BY FRESH AND WASHED CARROT SLICES

SUBSTRATE	FRESH TISSUE		WASHED TISSUE	
	GLUCOSE-1-C^{14}	GLUCOSE-6-C^{14}	GLUCOSE-1-C^{14}	GLUCOSE-6-C^{14}
C^{14} Supplied (CPM)	27,814	31,210	27,814	31,210
C^{14} Not absorbed (CPM)	1,170	1,300	855	975
% of Glucose absorbed	95	96	97	97
C^{14} in CO_2 (CPM)	5,560	2,760	16,450	6,210
C^{14} in Soluble fraction	11,700	15,500	3,670	8,350
C^{14} in Insoluble fraction	6,000	7,800	5,060	12,300
Total C^{14} recovered (CPM)	23,260	26,060	25,180	26,860

ANALYSIS OF TISSUES AFTER UTILIZING GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14}. The major features of comparison between fresh and washed slices are shown in table VIII. Virtually all the added glucose had been absorbed by the end of the experimental period. More than 60 % of C-1 and about 20 % of C-6 had appeared as CO_2 from the washed slices—considerably greater amounts of each than were produced from the fresh material. The converse is true both for the soluble and insoluble fractions. And while it is clear that in the fresh slices C-6 makes a bigger contribution than C-1 to these two fractions, the disparity is even greater in the washed slices.

In table IX the detailed analysis of washed slices is provided and the figures may be compared directly with table V in the previous paper (1). In washed slices C-1 and C-6 contribute equally to the soluble carbohydrate fraction, but the excess of C-6 is noticeable in the amino acid and organic acid components. Most of the carbon from C-6 not accounted for as CO_2 is found in the alcohol insoluble fraction. Unfortunately in this experiment complete breakdown of the alcohol insoluble residue was not achieved.

It is clear however that the protein component contains appreciable amounts of C-6. When the soluble carbohydrate and organic acid fractions were further fractionated (table X) the relative amounts of C^{14} in the various constitutents were similar in the samples derived from the differently labeled glucose. As in the fresh tissue there was no evidence whatsoever that C-6 entered any compound not also labeled (to a lower degree) by C-1. Again the amino acids were separated but the chromatograms did not contain sufficient C^{14} to give significant darkening on radioautograms exposed for 3 months.

TABLE X

C^{14} CONTENT OF COMPONENTS OF CARBOHYDRATE AND ACID FRACTIONS

SUBSTRATE	GLUCOSE-1-C^{14}	GLUCOSE-6-C^{14}
	C^{14} IN NAMED COMPONENT AS % OF TOTAL IN FRACTION	
Glucose	73	72
Fructose	12	8
Sucrose	14	20
Malic	57	63
Succinic	13	16
Citric & Isocitric	30	21

TABLE IX

DETAILED ANALYSIS OF WASHED CARROT SLICES AFTER SUPPLYING GLUCOSE-1-C^{14} OR GLUCOSE-6-C^{14} FOR 7 HOURS

SUBSTRATE	GLUCOSE-1-C^{14}		GLUCOSE-6-C^{14}	
	CPM	% OF C^{14} ABSORBED	CPM	% OF C^{14} ABSORBED
CO_2	16,450	61	6,210	21
Soluble carbohydrates	1,268	5	1,557	5
Amino acids	500	2	942	3
Organic acids	783	3	1,772	6
Alcohol-insoluble residue	5,060	19	12,300	41
Protein	1,350	5	2,655	9
Carbohydrate	820	3	1,328	4
Acids	314	1	613	2
Hexane Extract	990	3	2,700	9

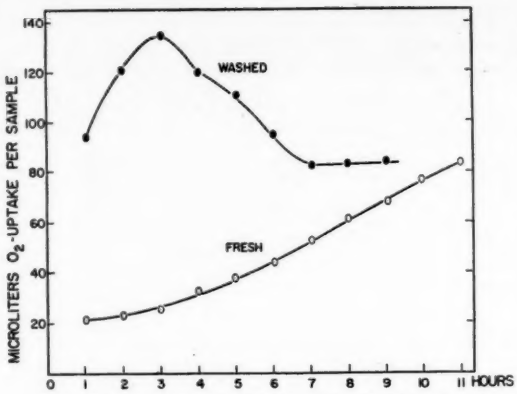

FIG. 4. Development of induced respiration in potato slices.

EXPERIMENTS WITH POTATO TISSUE: These experiments, while not so extensive as those with carrot, nevertheless indicate that similar changes occur in potato also. The development of the induced respiration is shown in figure 4 and the behavior of comparable washed slices during a subsequent 10 hour period is also shown. The respiratory increment as a percentage of the starting rate is more striking than that for carrots. Again after transfer to the respirometers a further respiratory rise is observed.

Experiments with labeled glucose on fresh potato slices are complicated by the fact that uptake is extremely slow during the first 6 hours and the yields of C^{14} in the CO_2 are therefore dangerously small. Nevertheless a definite decline in the C_6/C_1 ratio, beginning at 4 hours and persisting for several hours is noticeable (table XI). And in the washed material, as with carrot, the early ratios are significantly lower than for the fresh slices and show a steady rise in subsequent periods. Data on the uptake of glucose by fresh and washed slices are provided in table III.

The course of $C^{14}O_2$ release from specifically labeled glucose is shown in figure 5. Although the lag in glucose uptake has a distorting effect, it is clear that in fresh tissue the rate of release of C-3,4 exceeded that of C-1. It is equally clear that washing led to a tremendous increase in the yield from C-1 relative to that from C-3,4 and there can be no mistaking the superior contribution of C-1 in the washed tissue. At the end of 6 hours when the yield from C-6 was 27 %, and that from C-3,4 was 52 %, more than 70 % of C-1 had been converted to CO_2.

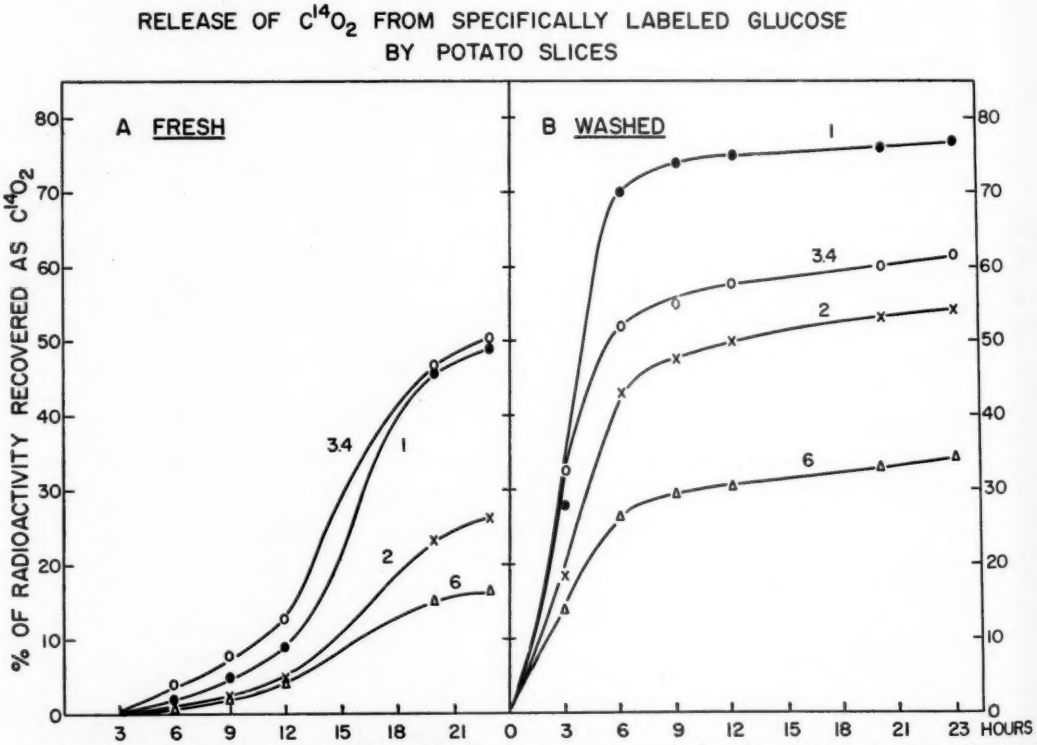

FIG. 5. Release of $C^{14}O_2$ from specifically labeled glucose samples by fresh and washed potato slices. Numbers on the curves refer to the position of C^{14} in the glucose provided.

TABLE XI

PROGRESSIVE CHANGES IN C_6/C_1 RATIO OF FRESHLY CUT AND WASHED SLICES OF POTATO

HR AFTER ADDING GLUCOSE	0-2	2-4	4-6	6-8	8-10	10-12	12-23	23-25
Fresh tissue	...	0.63	0.53	0.47	0.42	0.50	0.73	0.80
Washed tissue	0.38	0.41	0.38	0.82	1.1	1.1	1.4	1.4

Both fresh and washed potato tissue metabolized pyruvate (fig 6), but with this substrate also, the low metabolic rate of the fresh material and its poor ability to absorb metabolites may explain the relatively slow production of $C^{14}O_2$ by fresh tissue. The washed slices utilized the pyruvate quite avidly. As in all the other materials to which we have supplied specifically labeled pyruvates the carbons were released in order, but whereas C-1 was almost quantitatively recovered as CO_2, C-2 and particularly C-3 were indefinitely retained in the tissue, as a result of drainage of TCA cycle intermediates in synthetic events [Neal and Beevers (6)].

DISCUSSION

Washing slices of carrots in phosphate buffer led to developing an induced respiration the properties of which conformed to the general pattern described by Laties (3, 4). Within 3 hours of cutting the respiration rate of the slices began to rise steadily until it reached a value 2½ times that of the initial rate.

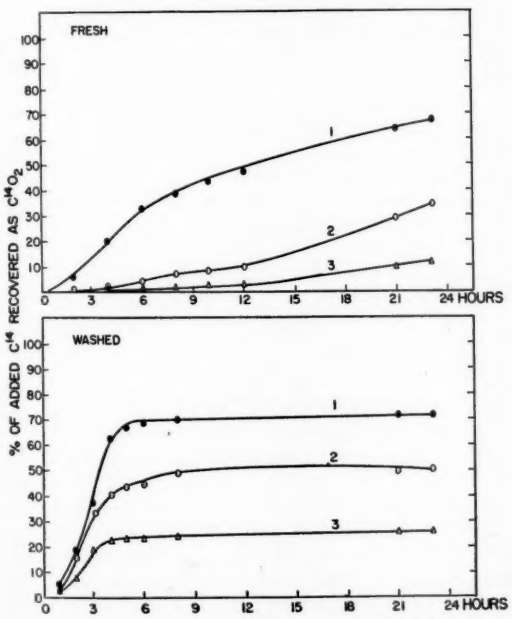

FIG. 6. Release of $C^{14}O_2$ from specifically labeled pyruvate samples by fresh and washed potato slices. Numbers on the curves refer to the position of C^{14} in the pyruvate provided.

This high rate was maintained for at least 24 hours. Once the induced respiration was fully developed, the rate of respiration was close to that of freshly cut tissue which had been treated with DNP. The marked contrast in the effects of DNP upon the oxygen uptake of fresh and washed tissue is similar to that described for potatoes by Sharpensteen (8). From these data it may be argued that, irrespective of the properties of the respiratory increment caused by DNP, one of the changes which took place while washing these tissues was an increased turnover of ATP and inorganic phosphate.

Development of the induced respiration of carrot slices was accompanied by a marked increase in the rate at which the slices absorbed glucose. The ability of slices of potato to absorb phosphate increased 200-fold during the development of the induced respiration (Loughman 5). Laties (4) has suggested that the induced respiration might be a necessary prerequisite for the performance of various types of cellular work in such tissue slices. The inhibitory effects of DNP, arsenite, and iodoacetate on the uptake of glucose by carrot tissue indicated that this was an active uptake. Our data do not allow us to decide whether or not this active uptake was entirely dependent upon the development of the induced respiration. However, there are good indications that freshly cut carrot tissue was capable of absorbing glucose. Within 3 hours of cutting, the carrot slices absorbed 15 to 20 % of the added glucose. Further, when glucose-1-C^{14} was supplied, at least 12 % of the activity absorbed during the first 3 hours appeared as $C^{14}O_2$ in that time.

There is good evidence that in the washed tissue, glucose was metabolized along the same pathways as have been described for freshly cut tissue. As might be expected from the higher rate of respiration, washing led to an increase in the rate of $C^{14}O_2$ production from labeled glucose and pyruvate. Nevertheless, the pattern of this release of $C^{14}O_2$, and, with one exception, the extent of this release were almost identical in fresh and washed tissue. The distribution of C^{14} from 1 and 6 labeled glucose which were supplied to washed tissue was similar to that described for fresh tissue. This similarity is good evidence that the glucose was metabolized through the same routes in both tissues. Finally, the pyruvate data, the appearance of activity from labeled glucose in malate, succinate, citrate, and isocitrate, the inhibition of oxygen uptake by arsenite all implicate the TCA cycle in the respiration of washed tissue. It is stressed that our data indicate that the participation of the TCA cycle in the respiration of carrot tissue was unaltered by development of the induced respiration.

Comparing the C_6/C_1 ratios of fresh and washed tissues revealed important differences between the two. When glucose was added to fresh tissue and the C_6/C_1 ratio was measured over the 24 hours which followed cutting the tissue, it was found that an initially low ratio was succeeded by values which were even lower. This decline in the C_6/C_1 ratio coincided with development of the induced respiration. Only when the induced respiration was fully developed did the ratio begin to rise. In contrast, when glucose was added to washed tissue the ratio showed the expected gradual increase from the initially low value. An even more striking difference was that the initial C_6/C_1 ratio of washed slices was only half that of freshly cut slices. This difference was due primarily to a disproportionate increase in the percentage of carbon 1, as opposed to carbon 6, of the added glucose which was converted to CO_2. This point is borne out more clearly when we consider the effect of washing upon the release of $C^{14}O_2$ from glucose-1-, -2-, -3,4- and -6-C^{14}. A most revealing feature is that the rate of conversion of C-1 of glucose to CO_2 after washing actually exceeded that for any other individual carbon in glucose.

In the washed tissue 45 % of the C^{14} from glucose-1-C^{14} was confined to $C^{14}O_2$. The comparable figure for freshly cut tissue was 28 %. The percentage of the C^{14} of 3-4, 2, and 6 labeled glucose which was released as $C^{14}O_2$ was similar in fresh and washed tissues.

The increased yield of $C^{14}O_2$ from glucose-1-C^{14} must be considered in the light of our knowledge of the pathways of glucose catabolism in carrot tissue. When this is done there can be no doubt that far more of the added glucose was immediately oxidized through the PP pathway in washed tissue than in freshly cut tissue. There also can be little doubt that this change was intimately associated with the induced respiration which developed while washing the tissue. The data strongly suggest that there was no such increase in the amount of the added glucose which was oxidized through the EMP pathway and that development of the induced respiration led to an increase in the proportion of glucose oxidized through the PP pathway relative to that oxidized via the EMP pathway.

The effects of washing upon the release of $C^{14}O_2$ from specifically labeled glucose and pyruvate confirm this hypothesis. If the increase in the amount of the added glucose oxidized through the PP pathway had been accompanied by a similar increase in the amount oxidized through the EMP pathway, then the contribution of labeled glucose to the pool of pyruvate would have been greatly increased. This would have been reflected either in an increase in the yield of $C^{14}O_2$ from 3-4, 2, and 6 labeled glucose or in an increase in the percentage of the carbon of pyruvate which was retained in the tissue. The development of the induced respiration did not lead to any marked increase in the yield of $C^{14}O_2$ from glucose -3,4-, -2-, or -6-C^{14}, nor was it accompanied by any change in the percentage of the added pyruvate which was retained in the tissue. Clearly there was no substantial increase in the amount of the added glucose which was metabolized through the EMP pathway. Thus we may conclude that in washed tissue a higher proportion of glucose oxidation was mediated by the PP pathway than in freshly cut tissue. The cause of this change is not clear. The demonstration that the C_6/C_1 ratio of freshly cut slices was unaffected by quadrupling the concentration of glucose (1) shows that the increased contribution of the PP pathway was not simply a result of the more rapid uptake of glucose by the washed tissue.

Our results indicate that the development of induced respiration in carrot tissue did not lead to any major qualitative change in the routes of glucose catabolism. On the other hand, we have shown that the contribution of the PP pathway to the induced respiration must have been very much greater than its contribution to the basal respiration. We have pointed out already (1) that it is not permissible to calculate from our data the exact contributions of the two pathways to the oxidation of glucose. Thus it is not possible to decide whether or not the induced respiration can be attributed entirely to the increase in the activity of the PP pathway. The fact that washing caused a far greater increase in the rate of oxygen uptake than in the rate of fermentation (table IV) supports, but does not prove this hypothesis.

The characteristics of the induced respiration in slices of potato have been well documented by Laties (3). In our experiments the rate of respiration increased fivefold. When compared on a basis of fresh weight the initial rate of respiration of potato slices was only one-half to one-third that of carrot slices prepared at the same time. This low rate of respiration was accompanied by a very slow appearance of $C^{14}O_2$ from the labeled substrates which were supplied to the potato slices. Only when the induced respiration had begun to develop was the percentage of the added activity recovered as $C^{14}O_2$ large enough to give reliable data. Nevertheless, development of the induced respiration was shown to lead to significant changes in the pattern of release of $C^{14}O_2$ from labeled glucose.

There are three strong indications that the development of induced respiration in slices of potato was also accompanied by an increase in the contribution of the PP pathway to the oxidation of glucose. First the C_6/C_1 ratio declined steadily during development of the induced respiration. Second, the initial C_6/C_1 ratio of washed slices was much smaller than that of freshly cut slices. Finally, washing led to a greatly increased yield of $C^{14}O_2$ from 1, 2, and 6 labeled glucose; in comparison the yield of $C^{14}O_2$ from glucose-3,4-C^{14} was increased only slightly. The fact that the release of $C^{14}O_2$ from glucose-1-C^{14} was increased far more than that from glucose-6-C^{14} implies that the proportion of glucose oxidized through the PP pathway was increased. The increased yields of $C^{14}O_2$ from 2 and 6 labeled glucose could be due to recycling of the pentose through the PP pathway.

Romberger and Norton (7) have presented preliminary evidence for operation of the PP pathway in freshly cut slices of potato. Our data suggest that the proportion of glucose which was oxidized via the PP pathway increased during the development of induced respiration and that in this respect slices of potato behaved similarly to carrot slices. Whether or not this is a general property of the induced respiration of tissue slices remains to be seen. It is clear, however, that in studying this problem we should no longer confine our attention to the TCA cycle and the mechanism of terminal oxidation.

Summary

Specifically labeled substrates (glucose and pyruvate) were used in a series of experiments to find if changes occurred in respiratory pathways from development of the induced respiration in carrot discs. The respiratory rise began about two hours after cutting and reached a value 2 to 2½ times that of the original rate in 9 hours. Coinciding with the beginning of the rise there was a distinctive change in the relative rates of release of carbons 1 and 6 of glucose as CO_2. The release of C-1 (which in the first 2 hr appeared at twice the rate of C-6) was accelerated more than that of C-6 during this period so that the C_6/C_1 ratio declined. Washed tissue, in which the induced respiration was fully developed, released C-1 at 4 to 5 times the rate of C-6 and the ratio showed the expected decline with time.

The abilities of washed and fresh tissues to convert individual carbons of glucose and pyruvate to CO_2 were compared. The rates of uptake of the metabolites were more rapid in washed tissue. Pyruvate was actively metabolized by both types of tissue, and in a similar fashion. C-1 was released quantitatively as CO_2 followed, after lag periods by C-2 and C-3 in order. Even after 24 hours there was considerable retention of C-2 and C-3. By contrast the pattern of release of the glucose carbons as CO_2 was different in the two types of tissue. In the fresh tissue the relative rates of release of the individual carbons of glucose was C-3,4 > C-1 > C-2 (=C-5) > C-6. In washed tissue the pattern was C-1 > C-3,4 > C-2 (=C-5) > C-6. Particular significance is attached to the distinctly superior contribution of C-1 to the CO_2 at a time when a considerable fraction of any C-1 entering pyruvate would be diverted to synthetic products. The changing pattern is ascribed to an increased participation of the pentose phosphate pathway as the induced respiration develops.

Analysis of tissues which had received glucose-1-C^{14} or glucose-6-C^{14} disclosed that a greater fraction of glucose was converted to products insoluble in alcohol (protein, polysaccharide) in washed tissue. However, as in the fresh tissue, although C-6 made a greater contribution than C-1 to each component other than CO_2, it did not appear in any component not also labeled by C-1. Thus although a greater fraction of glucose molecules were channelled through pentose phosphate the resulting 5-carbon unit is metabolized to pyruvate as in fresh tissue.

Experiments with potato discs indicate that in this tissue also, the pentose phosphate pathway makes an increasing contribution to glucose breakdown as the induced respiration develops.

Addendum

Recent observations (Hackett, D. P., D. W. Haas, S. K. Griffiths, and D. Niederpruem. 1960. Plant Physiol. 35: 8–9.) have shown that large increases in the activity of some enzymes concerned with electron transfer occur while washing potato discs. If these changes included an increased capacity for TPNH oxidation and this was the step originally limiting traffic through the PP pathway they may contribute to an explanation of our results. However, the onset of processes which consume TPNH in reductive events should also be considered (Butt, V. S. and H. Beevers. 1960. Biochem. Jour. xx: 76: 51 p.).

Acknowledgment

We record our appreciation to the Atomic Energy Commission for their financial support.

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PENETRATION OF MANNITOL INTO POTATO DISCS[1]

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This paper deals with a problem which, while minor in itself, may nevertheless loom large when interpreting experiments on the relationship between osmotic values and cell enlargement.

In 1953 Bonner et al (1) published some experiments on the uptake of water by slices of artichoke tuber (*Helianthus tuberosus* L.) which showed that the increase in respiration of this tissue caused by auxin was very much less when in hypertonic solutions of mannitol than when in water. However, Burström (6) gave reasons for believing that the value for the osmotic pressure of the tuber tissue at incipient plasmolysis, given by Bonner et al as 0.12 M, was too low, and reported a number of measurements which yielded values of 0.4 M and up. Further, while Burström's data undoubtedly established that the osmotic pressure of artichoke tissue was higher than had been thought, the data also brought out another, and a surprising, fact. This unexpected observation, which could not be explained at the time, was that when the tuber slices had been immersed in mannitol for 54 hours, the osmotic pressure at incipient plasmolysis had increased to extremely high values, in one case reaching 1 M and, in general, attaining values nearly proportional to the mannitol concentration used.

This great rise could mean one of two things; either mannitol steadily enters the cells, or else the exposure to mannitol causes liberation within the cells of some osmotically active material. Either of these two phenomena would have a most important bearing on studies of the water uptake of tuber slices which, since the pioneer work of Reinders (12), have proven to be useful material for the study of auxin action.

The result was of special interest to us since we had noted in several experiments with mannitol that potato discs, after their growth had been inhibited by high external osmotic pressure, tended to show a partial recovery. Examples of this will be shown below. More important, it was demonstrated (see fig 14 of ref 13) that if water uptake in auxin were restricted for 24 hours by adding 0.3 M mannitol, then on return for 4 hours to plain auxin the sections rapidly took up extra water and essentially recovered all the ground lost during the inhibition period. This was interpreted as evidence that auxin had been continuously modifying the cell walls at about the same rate as though the mannitol had not been present, i.e., that auxin action on cell walls had been uninterrupted. However, if mannitol had been entering the cells, this subsequent rapid water uptake might be the result merely of an increased osmotic value and would not be attributable to the action of auxin. Ordin et al (11) and Cleland and Bonner (7) have described comparable experiments with Avena coleoptile sections; the former authors concluded from plasmolytic measurements that the internal osmotic pressure of coleoptile tissue increases markedly in sucrose but only slightly in mannitol solutions. A further study of the recovery phenomenon in potato tissue therefore seemed important.

In this paper indirect evidence will first be presented which makes it improbable that mannitol enters the cells of potato tissue appreciably, and then direct measurements of mannitol uptake, using C^{14}-mannitol, will be described.

MATERIALS AND METHODS

From selected large potatoes, var. Katahdin, cylinders were cut lengthwise with a cork borer 9 or 15 mm in diameter and then sliced with a hand microtome into discs 1 mm thick[3]. The discs were vigorously washed in fast-running tap water for 15 minutes and then soaked in a shallow layer of distilled water (about 1.5 mm deep), for 24 hours. In a few cases, noted in the text, an osmoticum was used instead of water for this 24-hour period. The method was in all respects the same as that used in our previous studies of metabolism and water uptake of potato discs [see e.g. reference (10) and literature there cited] and very nearly the same as that used in most other laboratories. At 24 hours the slices were blotted with controlled pressure, weighed (initial fresh wt), and placed on plastic nets in petri dishes so that the discs just broke surface of the test solutions (cf. fig 1 of ref 10). The fresh weight after the experimental period was determined in the same way. In some cases a salt mixture comprising KCl : $CaCl_2$: : 3 : 1 was used as osmoticum. Its molarity was arbitrarily calculated assuming total dissociation. For this reason the concentration of the salt mixture, like that of Carbowax (see footnote to table II) is referred to as "M". Naphthalene-acetic acid, NAA, was the only auxin used, since it was found by Hackett and Thimann (10) and confirmed by Brian et al (4) that potato slices respond only very weakly to indoleacetic acid.

For radioactivity studies determinations were made

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[3] We wish to thank Dr. C. E. Cunningham of Maine Agricultural Experimental Station for a regular supply of high quality tubers.

TABLE I

PARTIAL RECOVERY OF POTATO DISCS FROM INHIBITION OF WATER UPTAKE BY MANNITOL (EXPT 30–53)

CONC OF NAA mg/l	CONC OF MANNITOL M	CHANGE IN FR WT, AS % OF INITIAL WT		
		2 DAYS	3 DAYS	5 DAYS
0	0	+9.31	+12.55	+12.14
0	0.17	+1.64	+0.82	+3.70
0	0.22	−6.00	−6.40	−2.80
0	0.27	−8.80	...	−3.20
10	0	+10.58	+20.77	+30.95
10	0.19	+2.02	+11.32	+24.65
10	0.24	*−6.42*	*+0.04*	*+15.25*
10	0.29	*−12.90*	*−7.25*	*+2.42*

only on the solution. The discs, usually 10 to 15 in a group, were removed from the experimental vessels and placed on nets as above, in contact with 0.3 M C^{14}-mannitol for various periods[4]. They were then taken out of the mannitol solution, thoroughly blotted and submerged in 10 ml distilled water with stirring; samples of 0.1 ml each were then withdrawn each 1 to 2 minutes for about 20 minutes, spread evenly on planchets, dried, and counted. The error due to self-absorption with minute quantities of substance (< 1 mg/cm² on the planchet) is negligible. When the sections were left in water for an hour or more in order to follow the slow outflow (cf. fig 3) the continued submergence caused general solute leakage to begin and self-absorption became appreciable; for this reason such experiments were not continued. The activity of the mannitol solution varied somewhat from one experiment to another; it was about 10^5 cpm per ml. Counts were made with a gas-flow windowless counter and corrected for background.

RESULTS AND DISCUSSION

I. OSMOTIC RECOVERY WHEN STILL IN OSMOTICUM. The change in weight of potato discs when immersed in concentrations of mannitol around 0.2 M is shown in table I, especially on the last two lines. At above 0.2 M there is a marked loss of water at first, then gain in weight begins. This we refer to herein as "osmotic recovery".

Table I shows that osmotic recovery in solutions without auxin is somewhat erratic and slow; it appears on the 5th day but not on the 3rd. In NAA, however, it appears clearly on the 3rd day in both of the higher concentrations used and is even more pronounced by the 5th day.

It should be pointed out that osmotic recovery was noted (albeit only to a small extent) in the Avena coleoptile in the very first publication on the use of mannitol in growth experiments (15). There the coleoptile sections which had shortened in 0.4 M mannitol in the first 37 hours elongated a little again after 36 hours more. Similar recovery can be detected in the recent data of Cleland (8), in which the irreversible elongation of Avena sections is shown to be promoted by auxin only when the mannitol is below a certain critical concentration. In that paper the different experiments in mannitol were carried out for different lengths of time. By measuring the critical mannitol concentrations carefully from Cleland's figures 2 through 8, which all present comparable experiments in 5 ppm IAA, we find the following:

TIME EXPOSED TO MANNITOL (HR)	CRITICAL CONCENTRATION (M)
3	0.073
4	0.125
6 (3 expts.)	0.113,0.125,0.127
10.5	0.178

The trend to increased critical concentration with increasing time in mannitol is clear, and confirms the fact that Avena sections also show some osmotic recovery.

Our first thought was of course that mannitol enters the cells and thus raises their suction pressure (DPD). A search was therefore made for another osmotic substance, sufficiently soluble in water to give hypertonic solutions, yet with a molecular weight so large that penetration into the cells could be ruled out. A satisfactory material was found in Carbowax. This polyethylene glycol is available in a series of molecular weights and is miscible with water in almost all proportions. One preparation was found toxic, but another, Carbowax 1500, was not. Its average molecular weight is 1,500 (indicated by the name) although the osmotic weight data below indicate that it contains an appreciable fraction of lower molecular weight. Nevertheless its entry into the cells seemed most improbable. Table II summarizes a growth experiment with this substance, in the presence and absence of NAA. Instead of being rinsed

TABLE II

PARTIAL RECOVERY OF POTATO DISCS FROM INHIBITION OF WATER UPTAKE BY CARBOWAX 1500 (EXPT 50–53)

CONC OF NAA mg/l	CONC OF CARBOWAX "M"*	CHANGE IN FR WT AS % OF INITIAL WT		
		AFTER 1 DAY	3 DAYS	4 DAYS
0	0	+7.1	+16.7	+20.9
0	0.156	*−4.6*	*+3.2*	*+6.0*
0	0.196	*−7.4*	*−3.2*	*−1.8*
10	0	...	+19.1	+36.8
10	0.156	*−5.5*	*+7.8*	*+13.2*
10	0.196	*−10.7*	*−2.8*	*−0.9*

* The concentration is given on the basis of a molecular weight of 1,500, but the fact that its osmotic effectiveness exceeds that of mannitol shows that a considerable fraction of it has a lower molecular weight.

[4] The mannitol was kindly supplied by Dr. H. S. Isbell of the National Bureau of Standards, Washington, D. C.

with water for 24 hours as usual, the discs were rinsed for only 15 minutes and then placed in the solutions shown. The osmotic recovery is shown best in the values underlined. Both with and without NAA it is clear that the tissues steadily recover the water content which they initially lost, the recovery being again improved by the presence of the NAA. On the whole, also, the rate and extent of osmotic recovery in Carbowax are not very different from those in mannitol.

II. Behavior of Tissue After Return to Water. Experiments in which tissues were placed in mannitol for some time and then returned to water (both phases containing auxin) were described for pea stem sections in 1951 (14) and for potato discs in 1954 (13). In the former case the mannitol-treated sections never did catch up to the controls; in the latter case they did. An experiment comparable to that given earlier (13), but in which the osmotic inhibition was more complete, is shown in detail in table III. Five groups of carefully matched discs, each group placed in two petri dishes of ten discs each, were prepared as usual; the maximum difference between the weights of these groups was less than 1 %. They were then allowed to grow for 2 days in NAA 10 mg per liter, and reweighed (top line of table III); their water uptake had been virtually identical. Three groups were now transferred to osmotica, one more group placed in fresh NAA solution as a control and the fifth group left dry on the nets but in a nearly saturated atmosphere. After 24 hours all the treated lots had lost weight except the one in the salt mixture, which had made a very small gain; the controls in NAA, however, had gained 190 mg.

All discs were now placed in fresh NAA. After 4 hours the large gain in weight of groups 2 to 5 brought them nearly up to the control level as previously described, and we can conclude, as in 1954 (13), that though growth in the osmotica had been temporarily abolished or nearly so, still the modification of the cell wall under the influence of the auxin had continued. The important point here is, however, that the discs that had been in mannitol or Carbowax did not, in fact, reach the control level exactly and in the subsequent 2 days they lagged considerably behind. By the 5th day the total gains (last line) show that the group that had been exposed to air recovered almost completely, but only those that had been in the salt mixture actually exceeded the controls in their final water uptake. If this be ascribed to a small amount of salt entry, causing an increased suction pressure, then by the same token neither the mannitol nor the Carbowax had entered the cells. Indeed, exposure to these two osmotica seems to have caused a permanent decrease in ability to grow. One explanation of this might be that deposition of cell wall material has continued during the exposure to these two osmotica, making the wall less extensible. However, such a process would be expected to continue also in the salts or in air (columns 4 & 5 of table III). The fact that it does not suggests a more specific explanation, namely that impregnation of the cell wall with mannitol or Carbowax may inhibit the enzyme system which enhances cell wall elasticity. (This might be due to the chemical relationship between these two substances and the carbohydrate of the wall). Cleland and Bonner (7) have also noticed, with Avena coleoptiles, that exposure of more than 3 hours to mannitol interferes with the subsequent growth.

Regardless of this small subsequent inhibition, however, there is certainly no indication in the present data of penetration into the cell by mannitol or Carbowax.

The rapid uptake of water on transference from the osmotica to water suggested that the suction pressure of the discs could be thus determined, by using a narrow range of osmotic solutions instead of water.

Table III

Behavior of Matched Potato Discs During & After 4 Hours in Osmotica or in Moist Air

Group	1	2	3	4	5
Wt on day 2	1.420	1.425	1.425	1.415	1.420
Next 24 hr in:	NAA	NAA + Carbo 0.17 "M"	NAA + Mann. 0.20 M	NAA + Salts 0.25 "M"	Moist Air
Wt on day 3	1.610	1.410	1.385	1.463	1.312
Wt after 4 hr in NAA	1.632	1.570	1.520	1.557	1.542
Wt on day 5	2.037	1.858	1.840	2.073	2.021
		% changes in wt			
Day 2–3, in osmotica	+13.6	−0.5	−3.8	+3.5	−7.4
In next 4 hr, in plain NAA	+1.5	+10.7	+10.4	+13.7	+16.2
In following 36 hr	+28.4	+20.1	+22.6	+29.4	+33.6
Total gain	43.5	30.3	29.2	46.6	42.4

* Note the complete return to control level after treatment with salts or after drying, but only partial return after treatment with mannitol or Carbowax. NAA 10 mg per liter in all solutions. Percentage change in weight calculated from weights on day 2. (Expt 46–53).

Indeed, this is essentially the standard classroom experiment. Accordingly a group of 5 discs after 3 days in osmoticum were blotted, weighed, and placed for a few hours in a series of salts or mannitol solutions of graded concentration, and then reweighed. Similar experiments have been used by Brauner and Hasman (2, 3) to compare potato discs which have been held in sugar and in Ca salts, or in air and in nitrogen. Unfortunately these workers (3) used for auxin IAA, which as mentioned above (see 10) has only very little effect on potato discs. NAA is a much more powerful auxin for this material. Nevertheless our results agree with Brauner's in principle. Figure 1A shows an example. The discs had been for 3 days on 0.25 M salt mixture, with or without NAA 10 mg per liter. In the first place the 1.5 hour measurement (solid line) shows that the solution in which the discs neither lose nor gain in weight (i.e., whose osmotic pressure is equal to the suction pressure) is considerably more concentrated for discs that have been on NAA than for the controls. The values are 0.282 and 0.230, respectively; hence *NAA has increased the suction pressure by 23 %*. Similarly, in figure 1B, in which the discs had been 3 days in Carbowax (0.12 "M") with Carbowax also used as the test osmoticum, the values are 0.151 and 0.126, i.e., *NAA has increased the suction pressure by 20 %*. The increase due to the auxin is thus nearly the same with two different osmotica.

Second, the 1.5 hour measurements in both figures show that the slopes of the lines are clearly steeper for discs kept in NAA than for controls. This is true for both gain and loss in weight. This demonstrates that

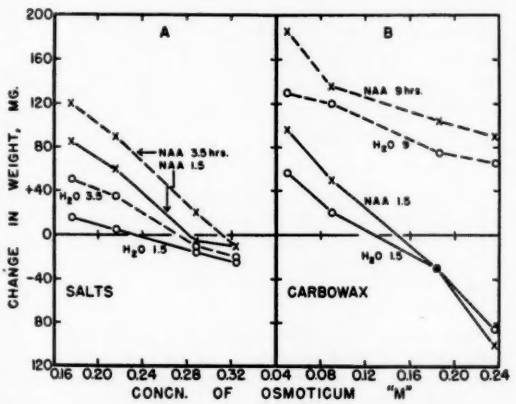


FIG. 1. Suction pressure determinations (gain or loss in wt) of groups of 5 potato discs. A: After floating for 3 days on 0.25 M KCl-$CaCl_2$ solution (3:1) with or without NAA 10 mg per liter. Solid lines, values after 1.5 hours in the test osmoticum; dashed lines, after 3.5 hours. The test osmoticum was the same as that for pre-treatment, i.e., KCl: $CaCl_2$: : 3:1. B: After floating similarly on 0.12 "M" Carbowax 1500. Solid lines, after 1.5 hours in the test osmoticum; dashed lines after 9 hours. The test osmoticum here was Carbowax 1500.

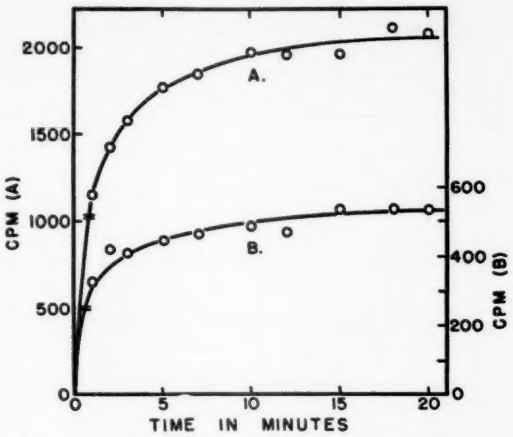


FIG. 2. Outflow into water of C^{14} from potato discs that had been floated on C^{14}-mannitol for 3 days (curve A) or 1½ hours (curve B). NAA, 10 mg per liter, was present in both cases Abscissa: isotope content in the water. The concentration of isotope in the mannitol was not the same in the two experiments.

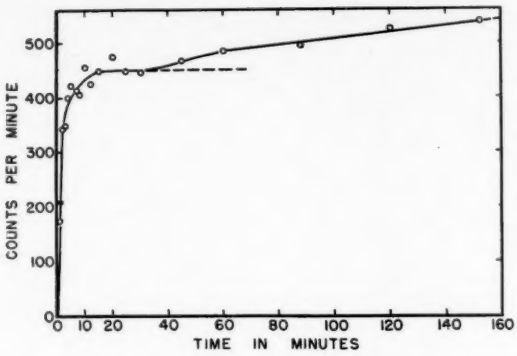
the elastic extension of the cell walls has been increased by the auxin. This result is particularly clear-cut because the discs during the 3-day treatment with osmoticum had grown very little; thus the increased elasticity can hardly be the *result* of growth.

In the third place, the figures show the osmotic recovery phenomenon very simply. The discs remain in the test osmoticum, except for being quickly blotted and weighed from time to time. Yet, as the dashed lines show, after 3.5 hours (fig 1A) or 9 hours (fig 1B) the discs which had previously lost weight now gained it. The change is in each case greater with NAA than without it (cf. tables I & II).

It may be well to emphasize that there appear to be three different responses involved: A, osmotic recovery, B, the effect of NAA in promoting this, C, the effect of NAA on cell wall elasticity.

III. OUTFLOW OF ISOTOPE AFTER EXPOSURE TO C^{14}-MANNITOL. The procedure of these experiments is described under Materials and Methods. After the discs had been kept for 3 days on NAA plus labeled mannitol, 0.3 M, they were carefully blotted and placed in stirred distilled water for 20 minutes, during which time 0.1 cc samples were removed every 1 to 2 minutes, dried down, and counted. In this way the outflow of absorbed mannitol into the solution could be followed. A typical result is plotted in curve A of figure 2. Equilibrium was reached within 20 minutes and removal to fresh solution at this time yielded no additional counts in another 20 minutes. The time taken to reach the half-maximum value was 0.9 minutes in figure 2 curve A, and in two similar runs the values found were 1.1 and 1.5 minutes, respectively.

The fact that so rapid an equilibration took place after 3 days in mannitol suggested that little real pene-



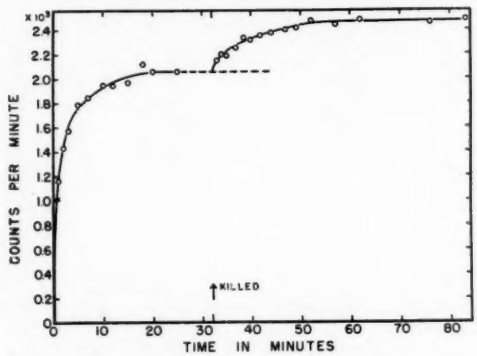


FIG. 3 (*above*). Outflow experiment similar to figure 2 but continued to 2½ hours. The discs had been 3 days on C^{14} mannitol containing NAA. Note the slow recommencement of outflow after 40 minutes' immersion.

FIG. 4 (*below*). Experiment similar to figure 3 but tissue killed by adding acetone after 32 minutes.

tration could have taken place. This was confirmed by exposing the discs to mannitol for shorter times. After 1 day in C^{14} mannitol (plus NAA) the outflow half-time was still 1.5 minutes. The outflow after only 1½ hours in C^{14} mannitol (again plus NAA) is shown in curve B of figure 2. The half-time was now about 0.5 minute, which may not be significantly different, the shape of the curve was the same, and again equilibration was complete in about 15 minutes.

The above experiments appear to indicate a one-compartment system for mannitol uptake, in which the mannitol is apparently taken up very quickly and is free to return rapidly to the external solution. However, if the discs remained in distilled water for more than 40 minutes it was found that a slow further release of isotope could be detected, indicating a small second compartment. Such behavior, in discs which had been 3 days on C^{14}-mannitol plus NAA, is shown in figure 3. Since the second compartment showed no sign of reaching equilibrium in 2½ hours, the cells in a comparable experiment were killed by adding acetone[5] after attaining their first equilibrium. Figure 4 shows that there was now a rapid release of isotope from the second compartment, the time for half-maximum value being about four minutes. The final activity of the solution was increased 430 cpm above the value of 2,050 cpm reached at the first equilibrium.

From the activities measured and the total volume of the discs (0.20 cc each) and of the solution (10.0 cc), the fraction of the volume of the disc into which the mannitol solution had entered can be calculated. Table IV summarizes these effective volumes for the two compartments in experiments of different duration. It may be assumed that the first compartment is the Apparent Free Space in the sense of Briggs (see 5) but that the acetone-sensitive compartment lies within the cells. It is evident that the effective volume of the free space does not change markedly with the time of exposure to mannitol, while the effective volume of the second compartment does. Thus the mannitol enters the free space very rapidly and the interior of the cells very slowly. The maximum concentration of mannitol reached in the tissue, as the sum of that in both compartments, in no case exceeds 0.09 M; that in the second compartment after one day does not exceed 0.008 M.

[5] Although acetone is not perhaps the ideal agent to destroy permeability its use was preferred to that of chloroform because of the undesirability of having a non-aqueous phase present.

TABLE IV

RATES AND VOLUMES OF OUTFLOW OF C^{14}-MANNITOL FROM POTATO DISCS AFTER DIFFERENT PERIODS OF EXPOSURE TO 0.3 M MANNITOL*

DURATION OF EXPOSURE TO MANNITOL	HALF-TIME IN MIN	EFFECTIVE VOLUME (AS % OF INITIAL VOL)			
		1ST COMPARTMENT*		2ND COMPARTMENT*	
3 days	1.1	27	25%	5.4	5.5 %
	1.5	21		5.7	
	0.8	27		5.5	
1 day	1.5	18	16.5 %	2.5	2.4 %
	...	15		2.2	
1.5 hr	0.5	18	18 %	0.75	0.75 %

* First compartment, outflow from intact tissue into water; second compartment, outflow after subsequently killing the tissue with acetone.

In a few of the experiments the discs which had been in contact with C^{14}-mannitol were kept in a closed vessel over KOH for an hour, but no significant quantity of $C^{14}O_2$ could be detected. This indicates that virtually no mannitol could have been dehydrogenated to fructose in the tissue, since otherwise this would have given rise to CO_2.

It can be concluded that no entry of mannitol takes place into potato tissue in a quantity sufficient to account for the osmotic recovery observed. It seems therefore that diminished turgor must give rise to the liberation of osmotic material within the cell, an effect strongly enhanced by auxin. This presumably means hydrolysis of a polymer, perhaps a reserve polysaccharide. It is at least a suggestive coincidence that another large polymer, namely RNA, has been reported to disappear when leaves are exposed to hypertonic solutions of mannitol (9). The significance of such a "manostatic" system, as a possible means of protection against the damaging effects of external osmotic changes, merits consideration in other cases where plants resist rapid changes in solute concentration.

Summary

Washed potato discs floated on hypertonic solutions of mannitol or a high molecular weight polymer of ethylene glycol (Carbowax 1500) lost weight at first and then, after periods between 3 hours and 3 days, gained weight again. This *osmotic recovery* is promoted by naphthalene-1-acetic acid, NAA.

That osmotic recovery could be due to entry of the osmotic material into the cells is improbable because: A, mannitol is known to enter plant cells excessively slowly, and the molecular weight of Carbowax, close to 1,500, should make its entry even slower; B, the subsequent enlargement of the discs in pure water does not indicate any permanently increased osmotic content, while, in contrast, discs that have been in a salt solution do show a slightly increased osmotic content.

A modification of the standard osmotic method for suction pressure determination shows that NAA increases the suction pressure of potato discs which have been exposed to hypertonic solutions. The same procedure allows a clearcut demonstration that NAA increases the elastic extension of the cell walls. This is further confirmation that one effect of auxin is exerted on the mechanical properties of the cell wall.

If the mannitol is labeled with C^{14}, the isotope taken up rapidly leaves the discs when they are returned to plain water, the time to reach half-equilibrium value being of the order of 1 minute, whether the discs have been on C^{14} mannitol for 3 days, 1 day, or 1½ hours. There is also a second, very slow, release of mannitol, which can be made rapid by killing the cells with acetone. The amount of mannitol in this "second compartment" does increase with increasing time of exposure to the mannitol, but even after 3 days it is barley 20 % of that which rapidly leaves the discs in water. After 1 day in 0.3 M mannitol a maximum of 0.008 M had entered the cells.

It is concluded that the mannitol in the first compartment is in the Apparent Free Space, which it can enter and leave rapidly, while that in the second (acetone-sensitive) compartment is within the cells. This result confirms those obtained by the osmotic methods in showing that very little externally-applied mannitol enters potato discs. The increase in the suction pressure of the discs, which appears as osmotic recovery, must therefore be due to a temporary liberation in the cells of osmotic material. This liberation, which is probably due to hydrolysis of a stored polymer, is caused in some way by the reduced turgor, and markedly promoted by auxin. The effect is separate from the increased cell wall elasticity caused by the auxin.

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FAT METABOLISM IN GERMINATING SOYBEANS
I. PHYSIOLOGY OF NATIVE FAT [1, 2, 3]

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The fat reserve of seeds constitutes a major source of reduced carbon for the growth of germinating embryos of many plant species, including the soybean, *Glycine max* (L.) Merr. Several investigators have attempted to correlate cytological and morphological observations with physiological and chemical changes during germination (11, 18, 19, 20, 23). It has been implied that esterases play a major role in fat degradation (2, 7, 12, 21, 31), but differences in solubility of lipids and the components of the aqueous cytoplasm make studies in such systems difficult. Evidence from electron-microscope studies of soybean native-fat suggests that in situ the fat may be enclosed in membranous structures [5]. These structures collapse as the fat is consumed. Membranes could provide both the hydrophilic and lipophilic properties required for fat metabolism and might be the loci of many of the enzymes involved.

Reserve lipids isolated centrifugally are microscopically similar to those observed in situ and appear to be homogeneous, uncontaminated by other organelles[5]. The lipid fraction isolated centrifugally was used in the present study. Physical, chemical, and biochemical changes occurring in this system during germination were investigated.

MATERIALS AND METHODS

Soybean seeds, lightly dusted with chloranil to retard fungal growth, were germinated in the dark at 21° C in 9 inch × 12 inch pyrex baking dishes, between sheets of absorbent tissue paper moistened with 200 ml of distilled water. After various periods of growth, the cotyledons were harvested, separated from seed coats, rinsed thoroughly with deionized water, and chilled.

Approximately 100 gram lots of cotyledons were ground in 200 ml of chilled grinding medium consisting of 0.5 M sucrose, 0.07 M tris [6], and 0.004 M EDTA, pH 7.3, for 2 minutes either in a Waring blendor or in a chilled mortar. The homogenate was passed through four layers of cheesecloth and centrifuged in the deep freeze at 20,000 × G for 15 minutes. The white fatty layer at the top of the liquid was carefully removed with a spatula, redispersed in 0.5 M sucrose, 1:20, and centrifuged at 28,000 × G for 20 minutes. The fatty layer was removed and redispersed in 0.5 M sucrose (1:5). This fraction is referred to as "native-fat." The washing procedure was omitted or repeated in certain instances as indicated in the data.

PHYSICAL MEASUREMENTS OF WASHED NATIVE-FAT: A: Rewashed native-fat was blotted with a piece of filter paper for fresh weight determination and then dried in the oven at 50° C to a constant dry weight. Weights were corrected for the occluded sucrose by the method of Trevelyan and Harrison (30). B: Light-scattering was measured spectrophotometrically at 520 mμ by the methods of Cleland (6) and Raaflaub (24). C: Viscosity was determined after dialysis in the cold to equilibrium against various sucrose concentrations. The corresponding dialyzates were considered as blanks. The determinations were carried out in an Ostwald viscometer at 28.3° C.

CHEMICAL ANALYSIS OF WASHED NATIVE-FAT: A: Lipid content was determined by extracting weighed samples of washed native-fat three times with ethanol: ether: chloroform (2:2:1). The solvents were evaporated from the pooled extracts, the residue representing total lipids. B: Lipid phosphorus was determined on ash solutions. For ashing, 50 ml of isopropanol and 0.5 g of $Mg(NO_3)_2$ were added per gram of lipid. The samples were mixed with the solvent and a piece of ashless cotton was added to the crucible and ignited. The ignition was completed with the aid of a strong flame from a bunsen burner [7]. The residue was taken up in dilute HCl and made to volume. Inorganic phosphorus

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[1] Received April 6, 1960.

[2] The work reported here was taken from a thesis submitted by the senior author to the graduate college of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Botany.

[3] Publication No. 340 of the U. S. Regional Soybean Laboratory, Urbana, Ill.

[4] Present address: Biology Dept., Johns Hopkins University, Baltimore, Md.

[5] R. F. Bils, personal communication.

[6] The following abbreviations are used: tris, trishydroxymethylaminomethane HCl; EDTA, ethylene diamine tetraacetic acid; TCA, trichloroacetic acid; DPN, diphosphoryridine nucleotide; ATP, adenosine triphosphate; RNA, ribonucleic acid; RNase, Ribonuclease; B.S.A., bovine serum albumin.

was determined by the method of Fiske and Subbarow (8). C: The residual pellet of the native-fat (after lipid extraction) was dissolved in 1 M KOH. Protein content of the pellet was determined by the method of Lowry et al (17) or by digestion with H_2SO_4 and nesslerization (14). D: Nucleic acids were determined by the methods of Ogur and Rosen (22).

Determinations of Enzymatic Activities Associated With Native-fat: A: *Lipase*: Washed native-fat was dispersed in water (0.5–2.0 ml) and incubated with or without 2 ml of 5% aqueous triacetin for 18 hours in a water bath at 36° to 38° C with continuous shaking. The initial pH of the reaction mixture was 7.3. The fatty acids released during incubation were titrated with 0.004 M standard NaOH. The increase in acid during incubation with triacetin, less the increase occurring in the absence of triacetin, was attributed to the activity of endogenous lipase on the added substrate. The products obtained in the absence of triacetin were partitioned between chloroform or petroleum ether and water and the fractions were titrated separately. The amino nitrogen liberated during self-digestion was determined by nesslerization of the supernatant after precipitating the incubation mixture with 10% TCA.

B: *Enzymes other than lipase*: An acetone powder of the native-fat was extracted for 30 minutes with 0.01 M potassium phosphate buffer, pH 7.0, (1:300 g/ml). The extract was cleared by centrifuging at 20,000 × G for 10 minutes. Ribonuclease activity was determined by measuring changes in RNA by the method of Ogur and Rosen (22). Ester phosphatase, ATPase, and pyrophosphatase activities were measured by the amount of inorganic phosphate liberated during incubation of 10 μ moles of substrate with the extract in the presence of 10 μ moles of $MgCl_2$. Lipoxidase activity was assayed by the modified thiocyanate method of Kock et al (15). Peroxidase was measured by the method of Lipetz and Galston (16). The presence of proteases was detected by NaOH titration after incubating the extract with 1% B.S.A., casein, or soybean protein. Dehydrogenase activity was measured by incubating 0.5 ml aliquots of the extract with 0.33 mg of DPN and the following substrates at 0.05 M final concentration, pH 7.0: potassium α-ketoglutarate, potassium lactate, glyceraldehyde-3-phosphate, and potassium β-hydroxybutyrate. The change in optical density at 340 mμ was read against blank tubes containing all components except the substrate.

Results

Physical Properties of Native-fat Fraction: *Water content*: The water content of native-fat decreased rapidly during 7 days of growth (fig 1). The

[7] This ashing method was suggested by F. I. Collins, U. S. Regional Soybean Lab.

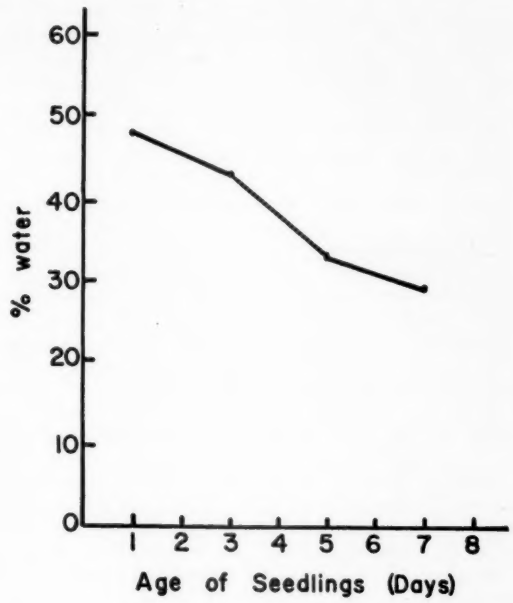


Fig. 1. Water content of washed native-fat as a function of age of soybean seedlings.

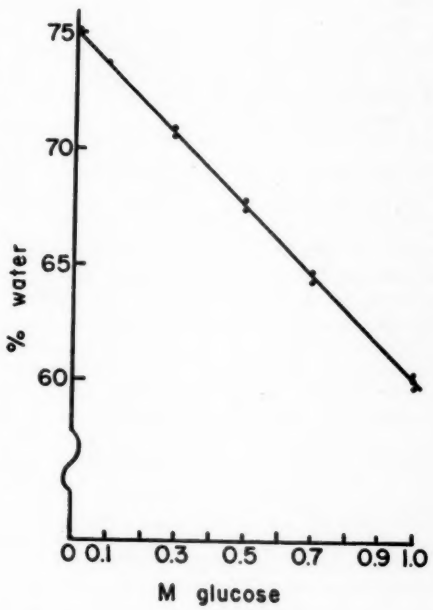


Fig. 2. The effect of glucose concentration on the water content of washed native-fat from cotyledons of 3-day-old soybean seedlings.

decrease was generally proportional to the decrease in protein (fig 4) indicating that the main portion of the water in the native-fat may be associated with protein as water of hydration. When the native-fat was dispersed in solutions of various glucose concentrations, the uptake of water by the native-fat decreased linearly with glucose concentration (fig 2). This may indicate the presence of semi-permeable membranes or of removal of water from hydrated surfaces in the native-fat.

Light-scattering: The optical density at 520 mμ decreased linearly with increasing sucrose or glucose concentrations, in contrast to what is commonly found in mitochondria (24). Changes in optical density are not necessarily an indication of volume change (28) but may also be due to changes in the refractive indices of either the particles or the dispersing medium (3). The change in optical density observed here was instantaneous following change in molarity of the medium. This seems to exclude the possibility of osmotic entry of water (6) so the change in optical density is attributed to changes in the relative refractive indices of the fat droplets and the suspending medium. Ball and Barnett (1) have reported similar results in experiments with an enzyme preparation of membranous and vesicular appearance from heart muscle.

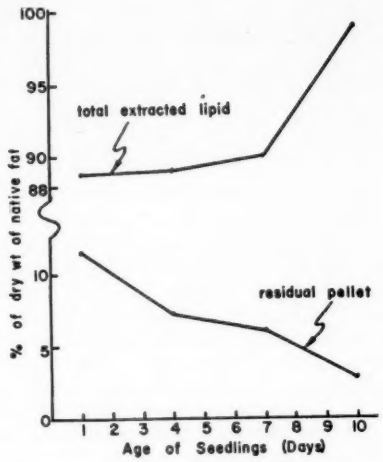
Relative viscosity: No significant change in the relative viscosity of native-fat dispersions resulted from change in osmotic strength of the suspending medium. Since relative viscosity is proportional to volume in Einstein's equation (13), the viscosity measurements, like those of light-scattering, did not provide evidence of a change in volume of particles following change in osmotic strength of the medium.

Sonic oscillation (10 kc/sec for 10–30 min), which is expected to disrupt structural membranes, did not affect the relative viscosity of native-fat dispersed in sucrose solutions.

Chemical Composition of Native-fat: The native-fat fraction contained mainly lipids, the relative amount of which increased during the experimental period (fig 3). The residual pellet, remaining after exhaustive extraction of the lipids, consisted mostly of protein plus traces of nucleic acids. Changes in amounts of nucleic acids, lipid phosphate, and protein with age are shown in figure 4.

Lipoproteins are known to be constituents of biological membranes (4, 26, 27, 29). Phospholipids are reported to be oriented at the surface of fat globules (25). If the fat were enclosed in membranes which were not degraded during germination, the ratio of lipid phosphate to protein should be constant throughout. Actually, protein and nucleic acids disappeared somewhat faster than lipid phosphate, suggesting that protein and nucleic acids are preferentially metabolized during germination.

Enzymatic Activities Associated With Native-fat Fraction: Incubation of the native-fat dispersions demonstrated the presence of lipase, which hydrolyzed the available endogenous substrate to produce free acids. This process is referred to as self-digestion. In addition, the lipase associated with the native-fat could hydrolyze added triacetin. The capacity for self-digestion increased threefold during 13 days of germination and growth. The specific activity of the native-fat on triacetin increased with repeated washings (fig 5), indicating that lipase was preferentially adsorbed on the native-fat while proteins other than lipase were effectively removed by the washing technique. Preferential adsorption of



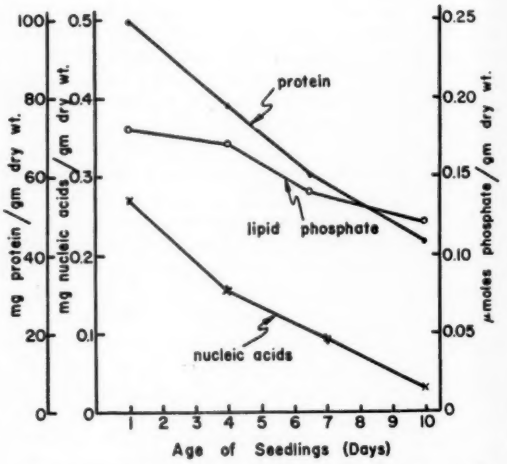

protein
lipid phosphate
nucleic acids
mg protein / gm dry wt.
mg nucleic acids / gm dry wt.
μmoles phosphate / gm dry wt.
Age of Seedlings (Days)

Fig. 3. Change in total extracted lipids and residual pellet of native-fat as a function of age. The native fat was extracted three times with ethanol : ether : chloroform (2 : 2 : 1).

Fig. 4. Chemical composition of native-fat as a function of age.

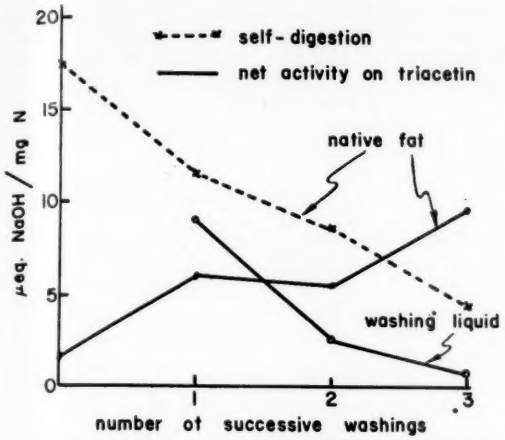


FIG. 5. Effect of successive washings of native-fat from cotyledons of 3-day-old soybean seedlings with 0.5 M sucrose (1 : 5 v/v) on its capacity for self-digestion and triacetin hydrolysis.

lipase was verified by dispersing the native-fat in a mixture of equal parts of B.S.A. (presumably an inert protein) and wheat germ lipase [8] (2.4 mg/ml each, pH 7.0). Upon reisolation of the native-fat it was found that the specific activity for self-digestion and for triacetin hydrolysis has been increased appreciably (table I). Activity on triacetin was also increased by dispersing native-fat in the supernatant fraction, (previously found to be associated with high esteratic activity) followed by reisolation. On the other hand, the capacity of native-fat for self-digestion decreased with repeated washings (fig 5).

The activity of endogenous lipase on triacetin was almost completely destroyed by sonic oscillation, while the capacity for self-digestion was increased (fig 6). The activity of wheat germ lipase on native-fat was essentially unaltered by sonic oscillation.

While the acids produced in self-digestion consisted of 85 to 90 % free fatty acids, about 10 to 15 % of the acid produced was amino acid.

Enzymes capable of degrading biological membranes were preincubated with native-fat. It was postulated that after enzymatic degradation of any limiting membrane, the fat would become more accessible as substrate for added lipase. The rate of self-digestion of native-fat (incubated for 18 hr at 37° C) was increased 1/3 by the action of *Crotalus adamanteus* venom, consisting mainly of phospholipases (1 mg of venom and 100 μmoles of $CaCl_2$ were

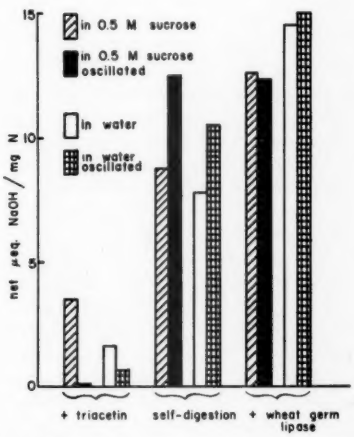


FIG. 6. Effect of sonic oscillation and tonicity on lipase activity associated with native-fat isolated from cotyledons of 3-day-old soybean seedlings. The native-fat was sonicated for 15 minutes at 10 kc/seconds in the cold. Wheat germ lipase : 4 mg/reaction mixture at pH 7. Incubation at 37° C for 18 hours.

[8] Worthington purified preparation.

TABLE I

PREFERENTIAL ADSORPTION OF LIPASE ON NATIVE-FAT OF SOYBEANS

	μ eq. NaOH/mg N					
	Washed native-fat	Supernatant	B.S.A. + Lipase	Native-fat passed through		% Increase
				Supernatant	B.S.A. + Lipase	
Activity on triacetin	8	3		14		75
	4		11		17	325
Self-digestion	37		13		85	130

A dispersion of native-fat was thoroughly shaken with either a solution of equal parts of wheat germ lipase and bovine serum albumin, 2.4 mg/ml each, or clear supernatant obtained after centrifuging a soybean cotyledon homogenate at 20,000 × G for 15 minutes. The native-fat was reisolated from the dispersion by centrifugation at 28,000 × G for 20 minutes. After reisolation, the native fat was incubated 18 hours at 37° C. Percent increase was calculated by comparing activity after passage with activity of washed native-fat in the first column.

TABLE II

ENHANCEMENT OF TRIACETIN AND NATIVE-FAT HYDROLYSIS BY AMINO ACIDS

ADDITIVE	SUBSTRATE	
	TRIACETIN	NATIVE-FAT
DL-Lysine	5.7	12.5
DL-Histidine	10.0	83.5
Glycine	0.2	5.6

DL-Lysine, 6.8×10^{-4} moles/ml, pH 8.5; DL-histidine, 6.4×10^{-4} moles/ml, pH 6.6; glycine, 1.3×10^{-3} moles/ml, pH 6.6 or 8.5. (Identical results were obtained at the two pH values). The reaction mixtures were incubated for 18 hours at 37° C. Results reported as net equivalents NaOH/mole amino acid added. For comparison, the activity of purified wheat germ lipase (2.5 mg/ml, pH 7.3) on triacetin and on washed native-fat was 2.6 and 2.0 net equivalents NaOH/mg protein, respectively.

added/3 ml of reaction mixture), 2/3, by that of trypsin (1 mg/3 ml of reaction mixture), but was not affected by adding RNase (1 mg/3 ml of reaction mixture). The activity of wheat germ lipase on native-fat (4 mg/3ml of reaction mixture) was not increased by preincubating native-fat with RNase, trypsin, or phospholipase A.

It has been reported that hydrolysis of triglycerides is enhanced by low-molecular-weight peptides or amino acids under certain pH conditions (5, 8, 9). Hydrolysis of the native-fat fraction was accelerated by histidine (pH 6.6), lysine (pH 8.5), or glycine (pH 6.6 or 8.5) in decreasing order of magnitude (table II). Thus, the amino acids produced during self-digestion may enhance fat hydrolysis.

A phosphate buffer extract of an acetone-powder preparation of native-fat was used as an enzyme source for detecting various enzymatic activities (table III). Several enzymes are associated with the native-fat. The association of these enzymes with the native-fat may be only superficial and due to contamination by other cytoplasmic components, but in view of the affinity of these enzymes for the native-fat they may be associated with it in situ.

DISCUSSION

An isolated system in which the condition of the fat is similar to that in situ is particularly advantageous for the study of fat metabolism because of the contrasting hydrophobic nature of the fat and hydrophilic properties of the surrounding cytoplasm. The isolated native-fat fraction is considered to resemble the fat in situ with regard to both the lipid material and the enzymes related to its metabolism, since electron-microscope studies indicate that in both the cotyledons and the native-fat fraction, fat bodies are surrounded by a limiting layer, possibly a membrane. The latter does not disappear in the course of germination but appears to collapse.

Physical measurements in the present study show that the fat bodies are limited by a hydrophilic layer from which water can be withdrawn osmotically. However, there is no evidence of a volume change, a fact suggesting either that there is no internal aqueous phase within the fat bodies or that the membrane does not possess semi-permeable properties. The rate of protein disappearance from the native-fat fraction is faster than that of phospholipids. This accelerated protein disappearance could be due to chemical alterations in the membrane or to superficial occlusion, or adsorption of proteins on the membrane (10). The latter would explain the high ratio of protein to phospholipid in the native-fat fraction as compared with membranous structures like mitochondria or nuclei.

TABLE III

ENZYMATIC ACTIVITIES, OTHER THAN LIPASE, ASSOCIATED WITH NATIVE-FAT FRACTION

ENZYMATIC ACTIVITY	SUBSTRATE	ACTIVITY/MG PROTEIN/30 MIN
RNAase	Yeast RNA	17 μg RNA Hydrolyzed
Ester phosphatase	Glucose-1-phosphate	0.120 μm P_i Released
	Fructose-1-6-diphosphate	None
ATPase	ATP	0.704 μm P_i Released
Pyrophosphatase	Pyrophosphate	0.710 μm P_i Released
Lipoxidase	Linolenic acid	0.045 μm Peroxide formed
Peroxidase	Peroxide derivative of linolenic acid	1.5 μm Peroxide disappeared
Protease	Bovine serum albumin	150 μeq Peptide bond hydrolyzed
	Casein	140 μeq Peptide bond hydrolyzed
	Soybean protein	162 μeq Peptide bond hydrolyzed
Dehydrogenase	Lactate	none
	α-Ketoglutarate	"
	Glyceraldehyde-3-phosphate	"
	β-Hydroxy butyrate	"

Reaction mixtures were incubated for 30 minutes at 37° C. Enzyme source was a 0.01 M phosphate buffer (pH 7.0) extract of an acetone-powder prepared from native fat.

Such occluded proteins may be functional enzymes whose close association with the fat bodies makes possible efficient interaction.

Endogenous and added lipase appear to be preferentially adsorbed on the native-fat bodies. The activity of the endogenous lipase can account for the major portion of the acids released during incubation of the native-fat fraction. The balance of the acids released during self-digestion is made up of amino acids, which may function to promote fat hydrolysis. Preincubating the native-fat with enzymes capable of digesting biological membranes, or physically rupturing the native-fat, does not render the fat more accessible to hydrolysis by added lipase. Apparently, the membrane, if it exists, does not introduce a chemical or physical hindrance to the hydrolytic action of lipase.

Enzymes such as RNase, protease, lipoxidase, peroxidase, and ester phosphatase are associated with the native-fat fraction. The presence of these enzymes may account for utilizing components such as protein, nucleic acid, and phospholipids in the native-fat fraction.

Summary

The metabolism of fat in germinating soybeans during periods up to 13 days was studied. Native-fat was prepared by homogenizing cotyledons in sucrose-TRIS-EDTA followed by centrifugation.

The native-fat initially contained about 50 % water but lost water during the growth period. The water is evidently held either osmotically or by adsorption, since the water content of fat suspended in glucose solutions was inversely proportional to glucose concentration.

The dry matter of native-fat initially consisted of about 90 % lipids and 10 % residual pellet. The latter was mostly protein but traces of nucleic acid were also present. After 10 days of germination, practically all the residual material had been lost.

The self-digestion activity of native-fat decreased with successive washings, but the specific activity on triacetin increased. Sonic oscillation destroyed activity on triacetin but enhanced self-digestion. Both self-digestion and activity on triacetin were increased by passing native-fat through a wheat germ lipase solution, indicating adsorption of lipase on the native-fat.

Several enzymes other than lipase were shown to be associated with the native-fat.

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MINERAL DEFICIENCY AND ORGANIC CONSTITUENTS IN TOBACCO PLANTS. I. ALKALOIDS, SUGARS, AND ORGANIC ACIDS[1]

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One of the most fundamental but least understood phases of plant physiology is the relationship between the quantities of the essential nutrient elements and the metabolic changes occurring in the plant. A living plant is a complex biological system composed of chemical constituents. Most minerals are essential building materials, and some form parts of indispensable catalysts. A deficiency in any mineral affects the normal metabolic system and thus disturbs the balance of the chemical constituents. Abnormal accumulation of certain compounds is associated with the development of a typical symptom usually recognized as due to the abnormal supply of a specific mineral.

In tobacco plants distinctive mineral deficiency symptoms were demonstrated by McMurtrey (7). The relationship of certain deficiencies to tobacco alkaloids and some organic fractions has also been reported (9, 10). It appears desirable for a definite pattern to be established between deficiencies of certain minerals and the organic constituents of that plant so as to provide basic knowledge.

This paper reports the relative differences in alkaloids, sugars, and organic acids in tobacco plants due to the deficiency of one of the following minerals: N, P, K, Ca, Mg, S, and B. This is a preliminary investigation of a semi-quantitative nature. More detailed and extensive studies are in progress and will be presented later.

[1] Received manuscript April 8, 1960.

Materials and Methods

Nicotiana tabacum L. var. Connecticut Broadleaf plants were grown in the greenhouse in 1957 (spring and fall), in 1958 (spring), and in 1959 (spring). Nutrient solutions, complete in or lacking N, P, K, Ca, Mg, S, or B, were prepared as described by McMurtrey (7). Two sources of nitrogen (nitrate and urea) were tested in Ca-deficient experiments.

Each seedling with five leaves and about four inches tall was grown first in the starter solution with half-strength complete nutrient. Three weeks later, after the plant was fully established, it was transferred to a partially deficient nutrient solution in a 10-liter jar. Deficiency symptoms usually were evident 5 to 8 days later. Calcium or boron-deficiency caused the drawn appearance of the tobacco plants because the buds had ceased to grow, which is referred to as "physiological" topping. As soon as the Ca- or B-deficiency symptom appeared, part of the corresponding control plants growing in complete nutrient were topped (decapitated), and suckers were removed thereafter. Plants were harvested 14 to 18 days after symptoms appeared, depending on the conditions of each crop season or the severity of the symptom.

There were four replications in each treatment. Composite samples of root, stem, and leaf from four plants were analyzed separately; the results were expressed on a per plant basis. All samples were extracted with acetone or ethanol in a Waring Blendor. Samples for alkaloid determinations were extracted with 50 % acetone and analyzed by both steam distillation and paper chromatography (11). A sepa-

rate portion of plant material was extracted with 70 % ethanol, and the residue after ethanol extraction was hydrolyzed with 4 N HCl. Both fractions were used for semi-quantitative determinations of sugars and organic acids by paper chromatography (2, 3, 6, 8).

Results and Discussion

In preliminary studies in 1957, alkaloid content of different mineral deficient plants agreed with published reports (9). In the order of decreasing amounts of total alkaloids, the results in spring crops were: topped, -B, -Ca, and control plants; and the results in the fall crop were: topped, -B, -Ca, -P, control, and -Mg plants. Although the effects of B-deficiency and Ca-deficiency upon alkaloid content are considered to be similar to those of manual topping, there is no generally accepted theory as to the exact effects of topping. Increased root branching resulting from topping is suggested as a possible explanation of alkaloid increase (9). However, the corresponding changes in leaf size and structure, coupled with increased physiological and biochemical activities, appear to deserve more attention.

Results obtained in 1958 and 1959 regarding the total alkaloid per plant from different treatments generally agreed well. Some variations in absolute quantity are due to the fact that 1959 experimental plants were put into deficient solutions 7 days earlier than 1958 plants, resulting in smaller plants in 1959 when deficiency symptoms appeared.

The nitrogen supply in each treatment, except that of N-deficient, was 2,500 mg including N from starter solution. The recoveries in 1958 and 1959 control plants were 2,039.9 and 1,843.9 mg, respectively, or 81.6 % and 73.8 %. In nitrogen-deficient plants the small amount of nitrogen found originated from the starter solution. All deficient and topped plants had a much lower nitrogen recovery than the nontopped control, although in some cases the alkaloid contents were much higher. In studying the relative percentage of alkaloid nitrogen to total nitrogen, topped, -B, and -Ca plants were among the highest, -P, -S, and -N plants were of medium rank, while -K, -Mg, and nontopped control plants were the lowest. It is clear that under deficiency conditions, the total amount of alkaloid formed is not a function of total nitrogen available or total nitrogen recovered in that particular plant. The withdrawal of certain mineral elements appeared to change the normal metabolic system and therefore to shift the balance of most, if not all, organic components, including the precursors for alkaloid formation.

Sugars and organic acids in 1959 samples only were studied. Sucrose, glucose, and fructose were found in the ethanol extract. Higher sugar concentrations (%) were observed in -Ca, -K, untopped control, and -N plants than other treatments while

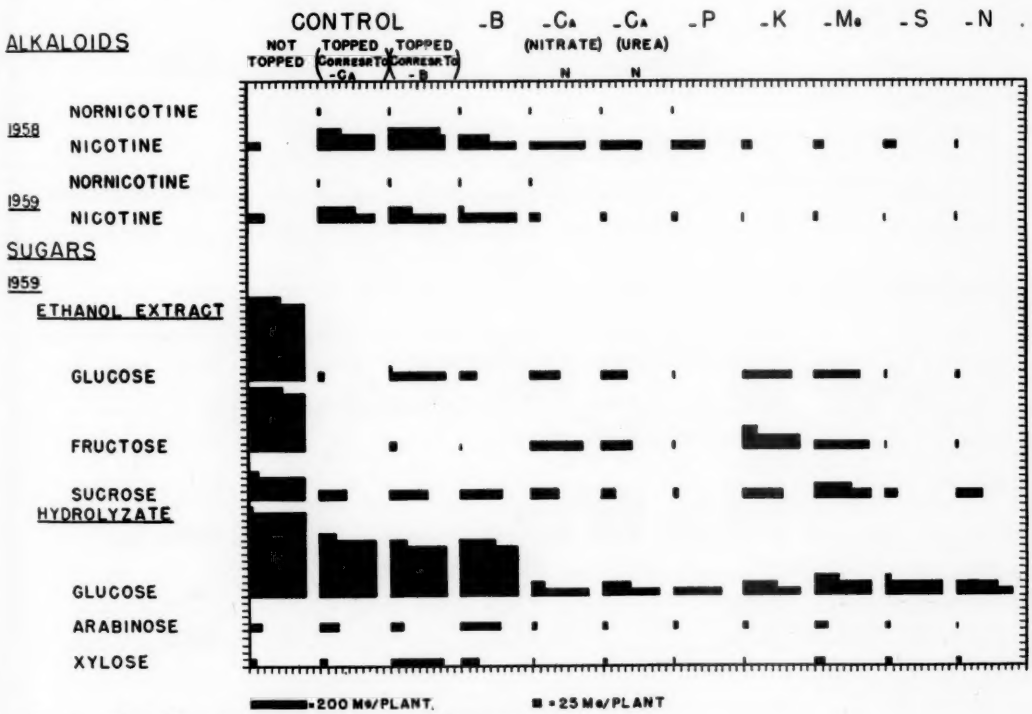

Fig. 1. Alkaloids and sugars in mineral deficient tobacco plants.

untopped control plants showed the highest total sugar per plant. Manual topping seemed to reduce sugar concentration tremendously; similar results were found in natural-topped -B plants. However, natural topping caused by -Ca did not change the sugar percentage in comparison with the control, although it decreased significantly the total sugar content.

In the hydrolyzate fraction glucose, arabinose, and xylose were found. All treatments except -Mg showed a higher sugar concentration than the untopped control, particularly -N, -Ca, and -S plants. Potassium- and boron-deficient plants and topped plants had a 40 to 100 % higher content of sugars than the untopped control. However, the untopped control plants were still the highest in total sugar content (per plant) followed by topped and -B plants, while -Ca and -P plants were among the lowest.

These results do not show the exact role which each element might play in the metabolism of sugars. It is clear, however, that manual topping had different effects on the content of sugars than did natural topping (-B, -Ca), although their effects on alkaloid content were somewhat similar.

Regarding organic acids, malic acid was found to be the most abundant; citric, glutaric, malonic, and ascorbic acid were also present in various quantities. Oxalic acid was not positively identified, probably because the special experimental procedure employed was not suitable to detect it. Aspartic and glutamic acids appeared in both organic acid and amino acid fractions, apparently due to incomplete separation. However, they remain major components in the organic acid fraction.

Malonic acid was found principally in ethanol extracts, while glutaric acid was found only in hydrolyzate. Glutamic acid appears to be the highest in ethanol extract; malic acid is the highest in hydrolyzate. In different mineral-deficiency treatments, the total organic acids per plant found in ethanol extracts in the order of decreasing amount is -B, -Mg, topped control corresponding to -Ca, topped control corresponding to -B, nontopped control, -P, -K, -S, -Ca with nitrate, -Ca with urea, and -N. In the hydrolyzates

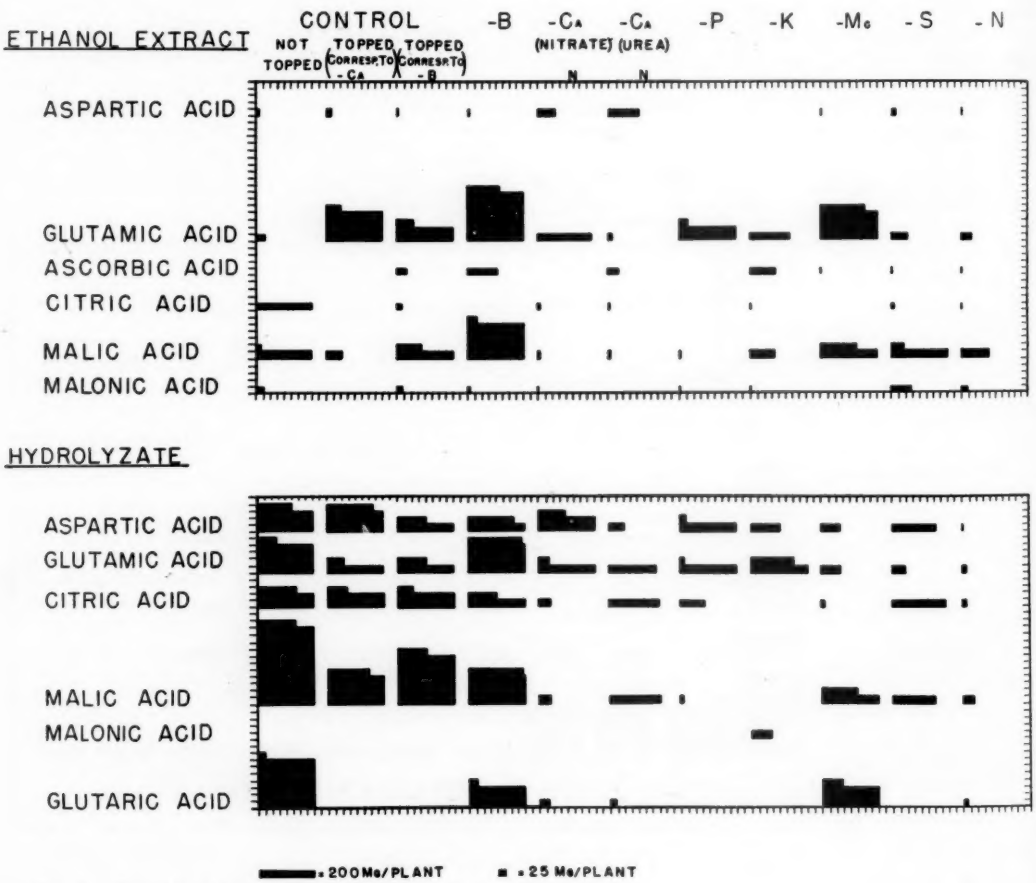

FIG. 2. Organic acids in mineral deficient tobacco plants (1959).

the order is nontopped control, -B, topped control corresponding to -B, topped control corresponding to -Ca, -Mg, -Ca with nitrate, -Ca with urea, -S, -K, -P, and -N. From the available data, the possible relation between alkaloid concentration and any particular organic acid will be discussed later; however, it is indicated that mineral deficiencies did cause major changes in the organic acid composition of tobacco plants.

In order to present a clear picture of these results, the relative differences of various organic groups are shown in figures. Figure 1 presents data concerning the contents of the different alkaloids and sugars of various mineral-deficient plants. Figure 2 lists the various organic acid contents. Figure 3 presents a comparison of total alkaloid, nitrogen, sugar, and organic acid contents.

In studying the relative proportion of alkaloid nitrogen to total nitrogen in each plant, the results showed that topped controls ranked the highest, followed by B- and Ca- deficient plants, and then other treatments. Manual decapitation (topping) appeared to stimulate utilization of a higher percentage of nitrogen into alkaloid formation than did physiological topping induced by Ca- or B- deficiency. This result may be interpreted in two ways. First, although manually or physiologically decapitated plants are all topped physically, the absence of Ca or B in physiologically topped plants theoretically causes more inbalance in the plant metabolic system than there is in plants which have Ca and B. Second, the absence of Ca or B caused severe leaf injury, generally known as the deficiency symptom, which affects the normal

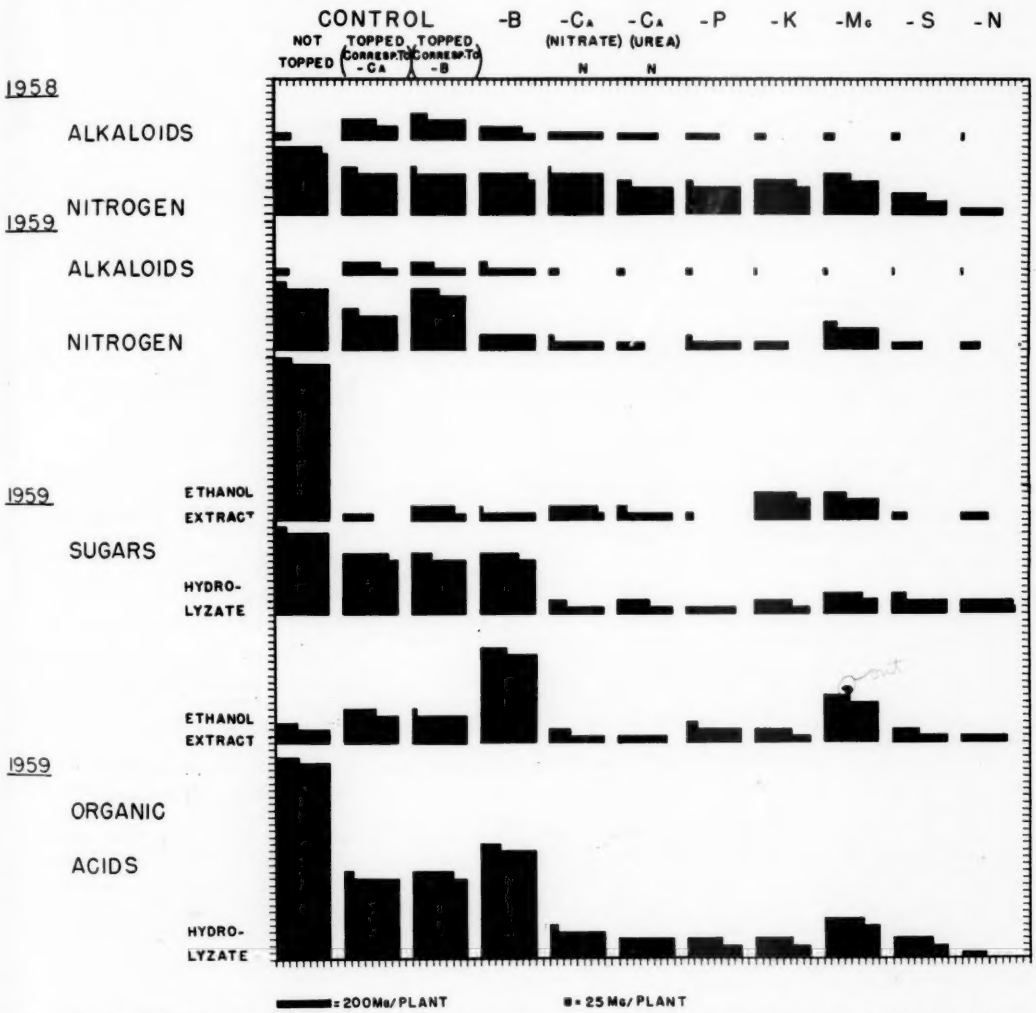

FIG. 3. Relative differences in total alkaloids, nitrogen, sugars, and organic acids in mineral deficient tobacco plants.

metabolic change as well as alkaloid formation. Other deficiencies showed some, but not such prominent reductions on alkaloid content.

In ethanol extracts, little sugar was found in -P, -S, -N, and topped control plants corresponding to -Ca ones. Nontopped control plants have the highest sugar content per plant among all tests, and only small amounts were found in plants under other treatments. A slight decrease in sugar content of the hydrolyzate fraction from manually-topped and -B plants in comparison with that from the nontopped control was observed. More decrease was found in plants of other treatments but not so wide a difference as that shown in free sugar fractions.

In general, sugar content is reduced by topping or mineral deficiency. The relation between sugar and alkaloid content, if any, is not clearly indicated here. Although it was shown that glucose may be used as an ultimate precursor for anabasine (1)—and in this experiment glucose is higher in topped and -B plants which have higher alkaloid contents than in those under other treatments—the relation is not consistent. Nontopped plants have considerably less alkaloids than the topped controls, but the former have a much higher glucose content.

The highest organic acid content in the ethanol extracts was found with boron-deficient plants (-B). In the hydrolyzate fraction, the organic acid content of -B plants is next only to the untopped control. These results are associated with the presence of a higher content of glutamic and malic acids in -B plants. Plants given several other treatments showed a higher malic acid content in the hydrolyzate fraction, but none had a higher glutamic acid content in either fraction than -B plants. This finding is interesting, since glutamic acid is believed to be a precursor for the pyrolidine ring of nicotine (4, 5); and B-deficient plants usually have a high nicotine content. However, why the manually-topped control plants have a lower glutamic acid content but a higher alkaloid content than -B plants must be explained.

Summary

A deficiency in any essential element affects the normal metabolic system and thus disturbs the balance of the chemical constituents of a living plant. This paper reports the relative differences in alkaloid, sugar, and organic acid contents in tobacco plants (*Nicotiana tabacum* L. var. Connecticut Broadleaf) due to the deficiency of one of the following elements: N, P, K, Ca, Mg, S, and B.

The data indicate the drastic changes in the plant metabolic system due to such deficiencies. Manually and physiologically (-B, -Ca) topped plants showed a higher alkaloid content than plants given any of the other treatments mentioned above. Alkaloid formation is considered not simply as a function of total nitrogen available or of total nitrogen content in a plant. Amounts of various sugars and organic acids in the ethanol extracts and hydrolyzate fractions of plants given different treatments were reported. Untopped control plants appeared to have a higher total sugar content than others. Glucose seemed to indicate some relation to alkaloid formation, but not a consistent one. Boron-deficient plants had a higher total organic acid content than untopped control plants, which are the next highest group among all treatments. The high glutamic acid content in -B plants and its possible role in alkaloid formation are discussed.

It is impossible to generalize the metabolic role of those elements studied from the limited data; however, the important effect of each element on the plant is clearly indicated.

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MINERAL DEFICIENCY AND ORGANIC CONSTITUENTS IN TOBACCO PLANTS. II. AMINO ACIDS[1]

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In a preceding paper (7) changes in several organic constituents (including alkaloids, sugars, and organic acids) in tobacco plants due to a mineral deficiency were reported. This paper is a continued discussion on this fundamental problem in plant physiology and deals with another important group of organic constituents, amino acids.

Amino acids have long been suspected of being related to alkaloid formation. Tryptophan, lysine, ornithine, proline, leucine, and many others were investigated as possible precursors of tobacco alkaloids (5). Recent reports (1, 3) indicated the possible role of glutamine and glutamic acid in alkaloid formation.

Results are presented here on relative changes of amino acids in tobacco plants due to the deficiency of one of the following elements: N,P,K,Ca,Mg,S, and B. Special attention is paid to the possible relation among amino acids and alkaloids which were reported in a previous paper (7).

MATERIALS AND METHODS

Plant materials were the same as those mentioned in the previous report (7). *Nicotiana tabacum* L. var. Connecticut Broadleaf plants were grown in different nutrient solutions. There were four replications in each treatment. Composite samples from four plants were analyzed but were expressed on a per plant basis. Different parts of the tobacco plants (root, stem, and leaf) were extracted with 70 % ethanol. The residue of ethanol extraction was hydrolyzed with 4 N HCl. Both fractions were used for amino acid study. The results are expressed on a per plant basis.

Amino acid content was determined by paper chromatography and was semi-quantitatively evaluated by comparison with the known amount of the standard developed in the same way. Two-dimensional, versatile paper chromatograph techniques described by Irreverre and Martin (2) were employed with some modifications. S & S 598 paper was used in a system consisting of tert-butyl-alcohol, formic acid, and water (70:15:15) in the first direction and tert-amyl-alcohol, 2,4-lutidine, and water (5:5:3) in the second direction, each for 18 hours. Ninhydrin was used for color development.

RESULTS

Results from 1958 and 1959 greenhouse experiments are presented here. The 1959 experimental plants were put into complete and deficient nutrient solutions 1 week earlier than in 1958; thus, the 1959 plants were smaller at the beginning and this resulted in the development of more severe deficiency symptoms. This difference is illustrated by the large variations existing in both the quantity and the compositions of amino acids in the 1958 crop as compared to the 1959 crop.

Alpha-alanine, aspartic acid, glutamic acid, glycine, leucine, isoleucine, proline, and serine were abundant. Phenylalanine, threonine, tryptophane, tyrosine, and valine were also present, but in lesser amounts. Beta-alanine, alpha and gamma-butyric acid, arginine, cysteic acid, cysteine, histidine, lysine, methionine, norleucine, ornithine, and tyramine were found in some samples in minute quantity. The presence of asparagine and glutamine was also noted.

Total amino acids per plant from different treatments are shown in figures 1 to 4. Figures 1 and 3 show results of free amino acids from the ethanol extracts, 1958 and 1959, respectively; figures 2 and 4 show the results from hydrolyzate fractions, 1958 and 1959, respectively. Corresponding alkaloid contents for the two years are also included in figures 1 and 3 in order to illustrate any possible relation between any amino acid and alkaloid.

DISCUSSION

The differences between 1958 and 1959 should be regarded as due to different treatments rather than variations between replications. The 1958 results were obtained under conditions where the withdrawal of certain elements took place when experimental plants were comparatively older than in 1959.

In general, free amino acids in tobacco plants increase when B, Ca, P, K, Mg, and S are deficient. The lowest amino acid content occurred in N-deficient plants. This agrees with the findings of Pleshkov and Fowden (4) for barley leaves.

The relative free amino acid content of topped control plants is closer to that of untopped control plants under deficiency treatments. This situation holds true even in the hydrolyzed fraction of the 1958 crop in which mineral deficiencies occurred late; but

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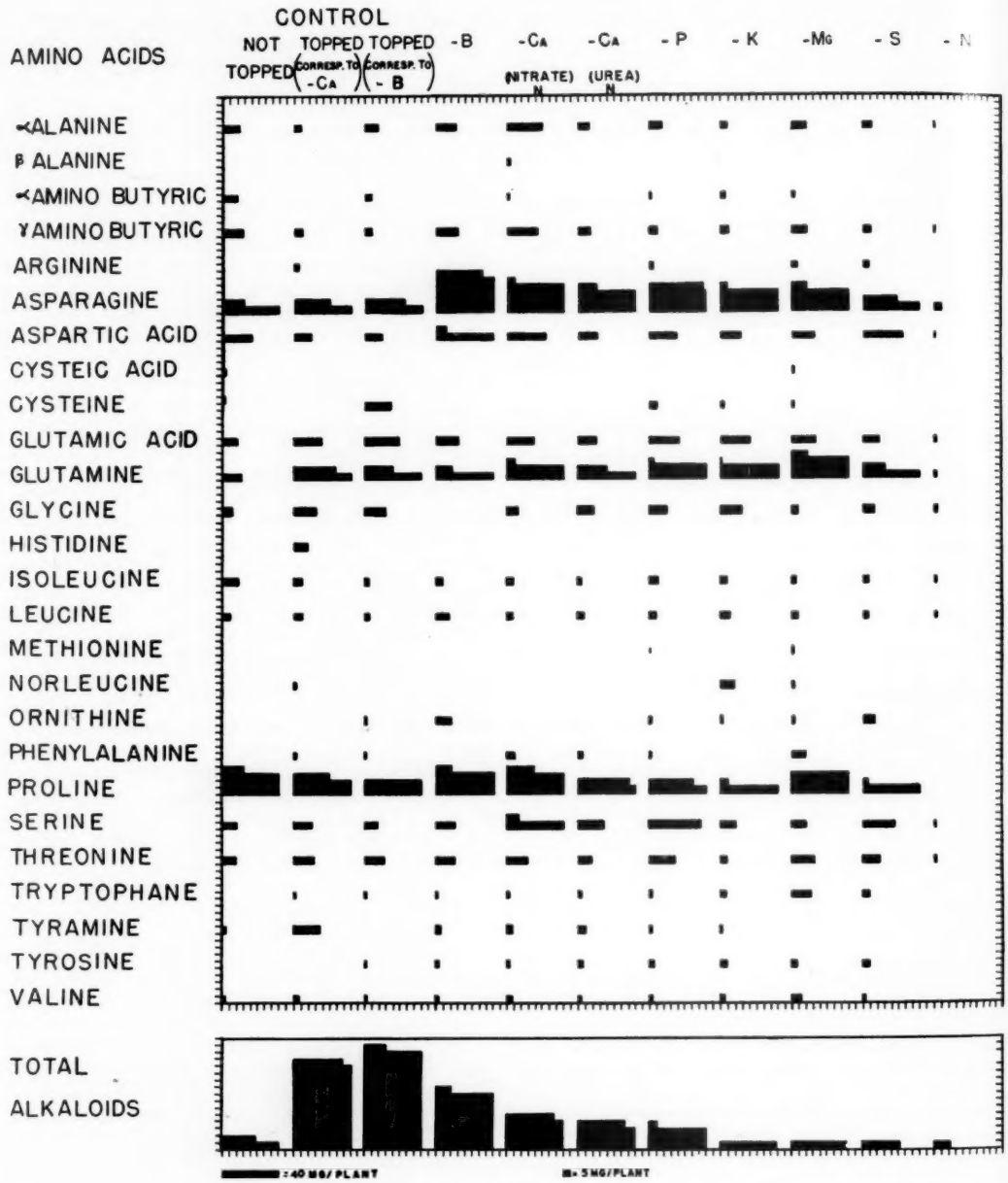

FIG. 1. Free amino acids in mineral deficient tobacco plants (1958).

it is not the case in the 1959 crop, in which deficiencies occurred earlier. It appeared that tobacco plant composition is not affected physiologically as much by topping as by a certain mineral deficiency.

The -Ca treatment, with urea as a source of nitrogen, had the highest free amino acid content (expressed as micrograms per gram of fresh material) in both years. In 1958 the order was -Ca with urea, -Ca with nitrate, -B,-P,-S,-K,-Mg, topped control corresponding to -Ca, topped control corresponding to -B, nontopped control, and -N. In 1959, the order was -Ca with urea, -S, -Ca with nitrate, -P,-K,-Mg,-B, topped control corresponding to -B, nontopped control, topped control corresponding to -Ca, and -N. Most of these differences were contributed by aspartic acid, glutamic acid, and their respective amides and also proline and serine. Boron deficient tobacco plants did not show an over-all high free tyramine content as reported for leaves by Steinberg (6).

Concerning combined amino acids obtained from hydrolyzed fractions, -Ca treatments were also among the highest, and -N, the lowest, in 2 years' observation. Results of hydrolyzed amino acid content from other treatments varied widely.

The control plants from these 2 years differed rather widely in their amino acid compositions. In 1958 -B and -Ca symptoms showed up on the same date, and the corresponding control plants were topped at the same time. In 1959, however, the Ca-deficiency symptom appeared 5 days ahead of the B-deficiency symptom; therefore, the control plants corresponding to -Ca were topped 5 days earlier than the control plants corresponding to -B. This situation would affect the composition of both the mineral defi-

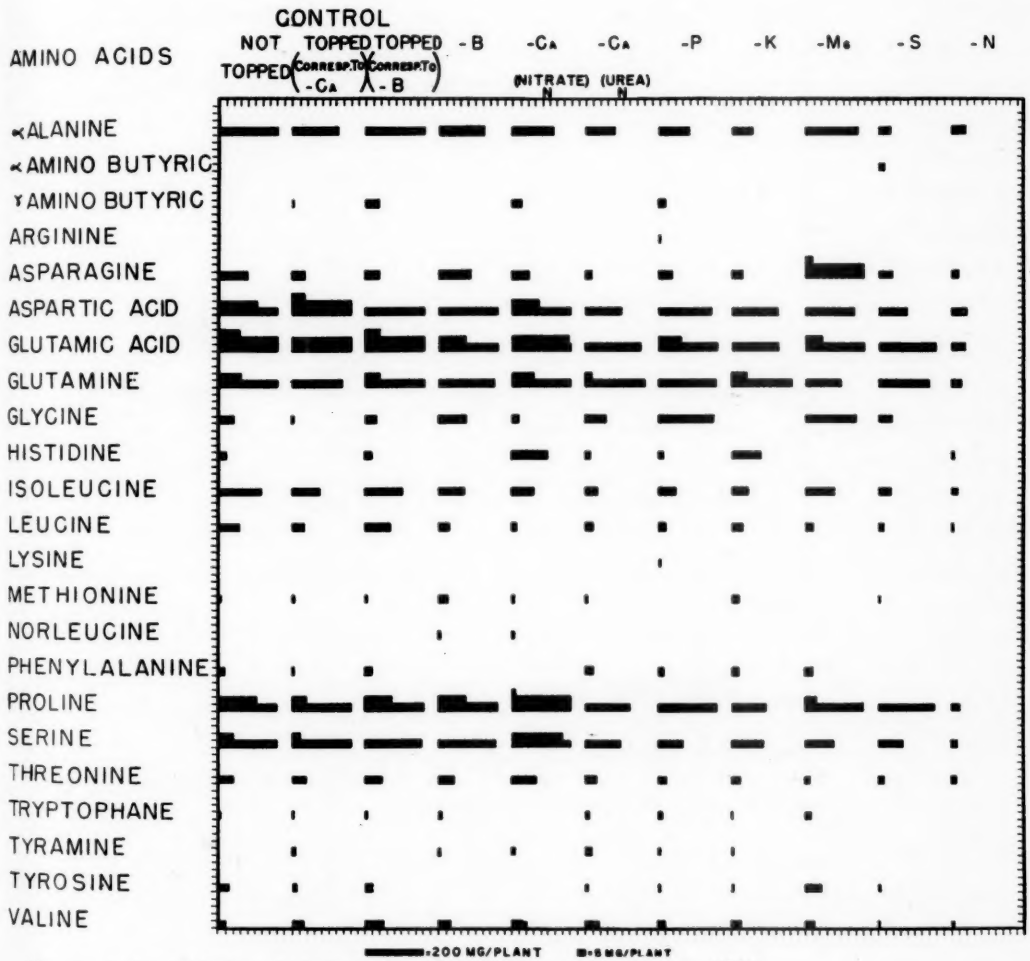

FIG. 2. Amino acids from hydrolyzate of mineral deficient tobacco plants (1958).

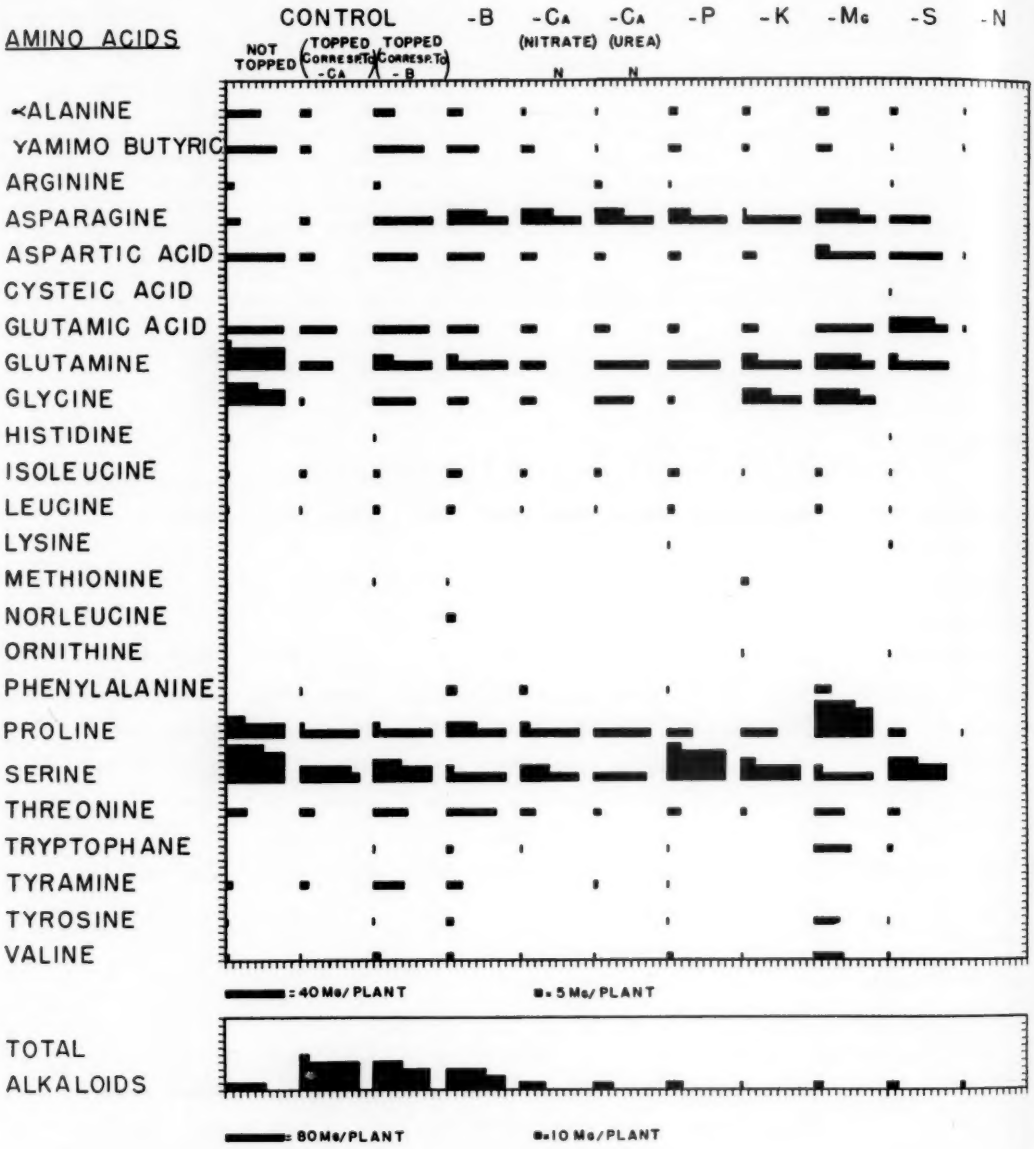

FIG. 3. Free amino acids in mineral deficient tobacco plants (1959).

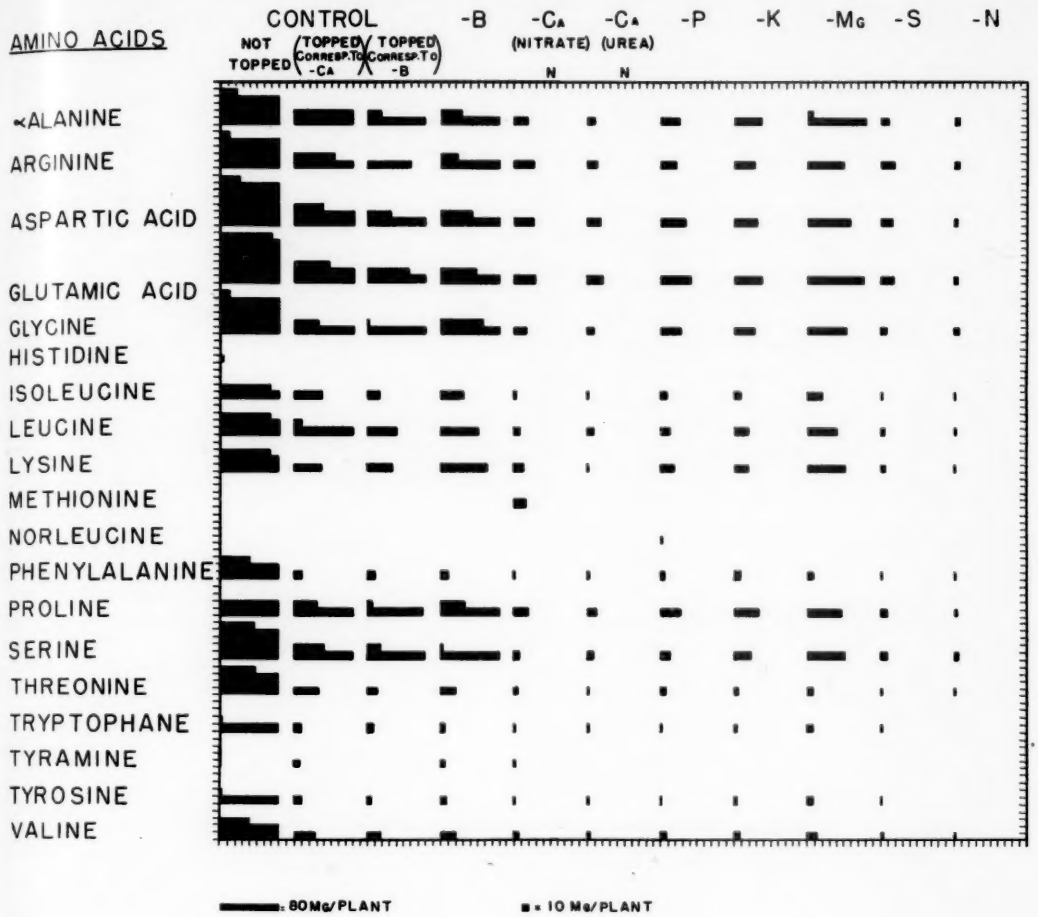

FIG. 4. Amino acids from hydrolyzate of mineral deficient tobacco plants (1959).

cient plants themselves, and also the corresponding topped control plants.

Concerning the total amounts of amino acid per plant, figures 1 to 4 clearly demonstrate the differences resulting from treatment. In the free amino acid fraction, little difference was observed in untopped and topped controls in 1958; but a higher amount of free amino acid was found in untopped controls than in topped ones in 1959. Among different amino acids and amides studied, the distribution of asparagine in each of the deficient plants indicated a close resemblance to the amount of alkaloid. Proline also had a similar distribution to alkaloids in all deficient plants except those minus Mg, which usually contained a higher content of amino acids either in free form or in the hydrolyzate.

The total amino acids per plant from the hydrolyzate showed few striking differences. Untopped control plants had a higher total amino acid content than topped ones in 1959, but not in 1958. Among mineral-deficient plants in 1959, -B and -Mg plants had a higher total amino acid content than others; but they had about the same as topped control plants, but the differences observed in the 1958 samples were not so prominent.

SUMMARY

The effect of mineral deficiency on the amino acid composition of tobacco plants is reported in this paper. An earlier withdrawal of certain elements during the plant growth period resulted in more severe symptoms

and more prominent effects on amino acid composition than later withdrawal.

In general, deficiency of B,Ca,P,K,Mg, and S caused an increase in free amino acid content in the plants, but not so in N-deficient ones. Less difference was observed in the amino acid content of the hydrolyzate fraction among mineral-deficient and control plants when deficiencies occurred at earlier stages of growth.

Considering each element, Ca and B deficiency appeared to have more effect than others. Mg also had a prominent effect on free amino acid content, but not in the hydrolyzate fraction.

Regarding the possible relation between free amino acid and alkaloids, the content of asparagine in the plants of each deficiency treatment appeared to show a positive relation to the alkaloid content in that treatment. A similar, but not so prominent, relationship between proline and alkaloid content was also indicated. Regarding glutamic acid, since the larger portion of it is included in organic acid fraction through ion-exchange process, the small amount left in the amino acid fraction showed no significant relationship with alkaloid content, although such relationship was indicated in the organic acid fraction in -B plants (7).

Additional observations are under way to study the change of chemical composition at different stages of the development of the deficiency symptom, and also at different leaf levels, in order to investigate the exact effect of such a deficiency.

Acknowledgments

We wish to express our thanks to Miss Mary Engelhaupt for the paper chromatography and evaluation of amino acids and also for drawing the figures. Thanks are also due Mr. Timothy LeFever, who did the earlier part of the analytical work.

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2-CHLOROETHYL TRIMETHYLAMMONIUM CHLORIDE AND RELATED COMPOUNDS AS PLANT GROWTH SUBSTANCES. V. GROWTH, FLOWERING, AND FRUITING RESPONSES AS RELATED TO THOSE INDUCED BY AUXIN AND GIBBERELLIN[1, 2]

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Several compounds closely related to choline and of the structure $CH_2X\text{-}CH_2\text{-}N^+(CH_3)_3 \cdot Cl^-$ are active as plant growth substances when X is a Cl, Br, or $=CH_2$ group (14). Treated plants develop thicker stems, shorter internodes, and intensely dark green leaves (7, 16, 17). The growth changes simulate those caused by exposure of plants to high light intensities, a short photoperiod, and low temperatures. They are opposite to, and more persistent than, those induced by gibberellin. With many plants a stepwise suppression in vegetative extension is possible. Slight, but significant increases in dry matter accumulation occur with tomato plants grown in solution cultures of 10^{-7} M 2-chloroethyl trimethylammonium chloride (17). Increasing amounts of the chemical cause corresponding reductions in vegetative extension. A consistent acceleration of flowering by tomato plants, however, is obtained over a rather wide range of concentrations which shorten stem length when applied to the root medium. This response is unlike that reported for any other plant growth substance.

These unique growth modifying characteristics, the marked specificity of structure for biological activity (14, 15), and the mutually antagonistic effects on vegetative overgrowth from applied gibberellin, suggested the need for further study on several well defined biological systems that respond to gibberellin or auxin. The gibberellins have now been established as naturally occurring plant hormones. Thus, any biochemical explanation of growth and flowering must involve both gibberellin and auxin. The modifying influences of 2-chloroethyl trimethylammonium chloride and related compounds on some classical auxin and gibberellin responses constitute the subject of this report.

MATERIALS AND METHODS

CHEMICALS: The compounds used in these studies consisted of 2-chloroethyl trimethylammonium chloride, 2-bromoethyl trimethylammonium bromide, allyl trimethylammonium chloride, and 2-chloroallyl trimethylammonium chloride. These were synthesized according to methods previously outlined (14). They were selected as the more active of the many compounds which have thus far been bioassayed. The potassium salt of gibberellin A_3 (Merck and Co., Rahway, N. J.) and 3-indoleacetic acid (California Corporation for Biochemical Research, Los Angeles), were used. Water solutions of all chemicals were prepared just before using.

PLANT MATERIALS: Grand Rapids lettuce (*Lactuca sativa* L.) was obtained as seed stock No. 7108 from the Ferry Morse Seed Co., Detroit, Mich., and was employed in all seed germination and photoperiodic studies. Brighton oats were used for the Avena coleoptile section growth tests. Thatcher wheat was used as in previous tests (16). The mutant dwarf-1 maize seed was supplied by Dr. B. O. Phinney, Department of Botany, University of California, Los Angeles. Biloxi soybeans [*Soja max* (L) Piper] were procured through Dr. R. W. Howell of the U. S. Regional Soybean Laboratories, University of Illinois, Urbana. Seeds of various dwarf and tall varieties of peas and beans were supplied by the Ferry Morse Seed Co. Fruit setting studies were conducted with the Michigan-Ohio Hybrid tomato (*Lycopersicon esculentum* Mill.) from seed supplied by Roy Burghart of Greenville, Mich.

GROWTH MEASUREMENTS: Lettuce seed was germinated in 9 cm petri dishes on Whatman No. 1 filter paper moistened with 5 ml of solution of the respective chemicals. Each treatment was replicated three times with 100 seeds per petri dish. All experiments were performed twice and the values were means of the two trials. Seeds were held in the dark for 72 hours at a temperature of 26° C. For the different periods of red light exposure a source with energy emitted primarily in the 6,500 to 6,600 A region was used. High energies of irradiances were obtained from a red coated incandescent lamp. Exposures of the seeds to red light were initiated 10 minutes after the seeds were placed in contact with the various solutions.

The procedure for growing and sectioning Avena coleoptiles was that described by Leopold (6). Coleoptiles 2 to 3 cm in length were selected and a 5 mm section cut 3 mm below the tip. The sections, with the leaf enclosed, were floated five to a dish in 20 ml

[1] Received for publication April 11, 1950.

[2] Journal Article No. 2610 of the Michigan Agricultural Experiment Station.

of the test solutions which contained, in addition to the chemical to be tested, a phosphate-citrate buffer (pH 5.0) and 3 % sucrose (13). Single treatments were replicated three times. After 48 hours at 25° C the lengths of the sections were measured to the nearest 0.1 mm.

Seedlings of the mutant dwarf-1 maize (12) were grown in 6 inch clay pots of soil in a greenhouse maintained at a night temperature of 20° C. Approximately 12 days after the seed was sown the normal plants were removed. This left, in each pot, three to six dwarf seedlings each of which had developed its first leaf. At this time 300 ml of the designated molar concentrations of the various chemicals were applied to each pot as a soil drench. After 15 days the lengths of the second leaf sheaths were measured. Treatments were replicated three times, and a single pot of three to six seedlings constituted a replicate.

Photoperiodic experiments with the Biloxi soybeans and Grand Rapids lettuce were conducted in a greenhouse maintained at a night temperature of 18° C. Plants were grown in standard 6 inch clay pots. Photoperiods of 9 and 18 hours were maintained from seedling emergence until flowering. Chemicals were applied as a soil drench of 300 ml of the designated molar solutions per pot. The initial treatment for soybeans was 2 to 3 days after seedling emergence. Lettuce was treated after six to eight true leaves had formed. The treatments for both soybeans and lettuce were repeated after 2 weeks. Chemicals were similarly applied in other greenhouse tests with dwarf and tall varieties of peas and beans.

For comparative growth responses of peas, beans, and wheat in the light and dark, seeds were planted in 4 inch clay pots of dry vermiculite or sand. Solutions of the various chemicals were then applied as 100 ml aliquots to each pot. For tests with peas and beans a constant temperature of 25° C was maintained. Light exposed plants were subjected to 12 hours of light daily. This was supplied by T/12 cool white fluorescent tubes with an illuminance of approximately 200 foot candles at the plant level. Total plant heights were recorded after 12 days. Similar procedures were used in the wheat experiments.

Promotion of parthenocarpic fruit growth of the tomato was determined by Luckwill's procedure (9). Three flowers of the first cluster were emasculated just prior to anthesis and 0.01 ml of the respective solutions was applied directly to the ovary. Three single plant replicates comprised each treatment. Ovary diameters were measured after 12 days.

STATISTICAL: F tests for differences between treatment means were expressed as probabilities of significance. Exceptions were made where differences were obviously significant without statistical verification.

RESULTS

GERMINATION OF LETTUCE SEED: The germination percentage of certain lots of Grand Rapids lettuce seed in the dark ranged from 21 to 23 %. There was a progressive increase in seed germination, approaching 100 %, with increases in either durations of light exposure or molar concentrations of gibberellin (table I). The percent germination of lettuce seed was strongly inhibited by 2-chloroethyl trimethylammonium chloride and related compounds. Adding allyl trimethylammonium chloride caused a marked reduction in germination as concentrations were increased from 10^{-3} to 2×10^{-2} M. This compound also antagonized the stimulation of germination by both red light and gibberellin. Reversal of the red light response was greater than for that induced by gibberellin. Concentrations of 10^{-4} M, or less, of

TABLE I

INHIBITORY EFFECTS OF ALLYL TRIMETHYLAMMONIUM CHLORIDE ON GERMINATION OF GRAND RAPIDS LETTUCE SEED IN DARK AFTER EXPOSURE TO VARIOUS INCREMENTS OF RED LIGHT, AND IN PRESENCE OF VARIOUS CONCENTRATIONS OF GIBBERELLIN

ALLYL TRIMETHYL-AMMONIUM CHLORIDE	EXPOSURE TO RED LIGHT (MIN)					
	0	0.17	0.5	1.0	5	20
Molar			*% Germination*			
0	21	27	36	45	82	96
10^{-3}	14	10	18	18	25	55
10^{-2}	7	6	9	14	16	36
2×10^{-2}	0	0	0	0	0	14
			Gibberellin (molar)			
	0	*10^{-8}*	*10^{-7}*	*10^{-6}*	*10^{-5}*	*10^{-4}*
0	23	28	32	41	60	97
10^{-3}	15	16	25	26	55	87
10^{-2}	13	14	19	22	42	77
2×10^{-2}	0	1	2	4	14	42

TABLE II

EFFECTS OF ALLYL TRIMETHYLAMMONIUM CHLORIDE ON STRAIGHT GROWTH OF AVENA SECTIONS AND ITS MODIFYING EFFECTS ON GROWTH RESPONSES INDUCED BY 3-INDOLEACETIC ACID AND GIBBERELLIN*

ALLYL TRIMETHYL-AMMONIUM CHLORIDE	3-INDOLEACETIC ACID (MOLAR CONC)				GIBBERELLIN, 3-INDOLEACETIC ACID
	0	10^{-7}	10^{-6}	10^{-5}	10^{-5}, 10^{-5}
(*Molar*)					
0	10.9	13.2	14.2	15.0	16.3
10^{-4}	10.8	14.2	15.8	16.1	16.7
10^{-3}	10.5	13.1	13.7	14.5	15.8
10^{-2}	9.8	8.7	9.7	10.4	14.3
2×10^{-2}	7.8	7.6	8.2	8.5	10.7
5×10^{-2}	7.4	6.7	6.9	7.7	9.1

* The values are the final length in mm of 5 mm sections after 48 hours in the various solutions at 25° C. All solutions contained phosphate-citrate buffer (pH 5.0) plus 3 % sucrose.
Least differences necessary for significance between any two values: 0.05 = 1.5, 0.01 = 2.0

allyl trimethylammonium chloride had no consistent effect on germination when used alone or in combination with gibberellin.

The marked inhibition from 2×10^{-2} M allyl trimethylammonium chloride was not an osmotic effect. Germination of lettuce seed was only slightly suppressed by controls of 2×10^{-2} M solutions of HCl or NaCl which were considerably below those concentrations having an osmotic effect (5). Similar controls (KCl or NaCl) carried in all subsequent experiments gave no biological response.

AVENA COLEOPTILE SECTION GROWTH: The responses of sectioned plant materials were first investigated as a possible bio-assay for the growth regulating properties of the many active derivatives of 2-chloroethyl trimethylammonium chloride that have been synthesized (14, 15). Stem and hypocotyl sections of bean and tomato seedlings, however, were less sensitive than the intact plants. Avena coleoptile sections were not significantly depressed in growth unless incubated in solutions that exceeded a concentration of 10^{-3} M (table II). The typical Avena section growth response to 3-indoleacetic acid (IAA) may also be noted along with the slightly increased growth when gibberellin was added. Allyl trimethylammonium chloride at concentrations exceeding 10^{-3} M inhibited the growth of avena sections in the presence of IAA. In fact these chemicals completely suppressed the IAA growth stimulation of Avena sections. When gibberellin at 10^{-5} M was incorporated with the highest concentration (10^{-5} M) of IAA, the suppression of elongation by high levels of allyl trimethylammonium chloride was much less. Straight growth of Avena sections was only moderately increased when gibberellin was added in combination with IAA. Gibberellin, however, markedly reduced the growth suppressing effects of allyl trimethylammonium chloride. This antagonistic effect of gibberellin on the growth retarding effects of allyl trimethylammonium chloride in the presence of IAA was in accord with that observed on intact plants and in lettuce seed germination where an endogenous source of auxin was present.

GROWTH OF DWARF PLANTS: Vegetative extension in dwarf as well as tall varieties of plants was reduced following treatment with 2-chloroethyl trimethylammonium chloride and related compounds. Genetic dwarfism was accentuated. This was illustrated by the length of the second leaf sheath of mutant dwarf-1 maize (table III). Growth suppression was proportional to concentration. The expected extension of the leaf sheath occurred with gibberellin. When the two chemicals were combined, the response to gibberellin was not significantly altered. The results on corn dwarfs were not conclusive in demonstrating an antagonism between the two chemicals. There was, however, evidence in test II of growth promotion from 2-bromoethyl trimethylammonium bromide in combination with 10^{-4} to 10^{-5} M gibberellin.

GROWTH IN LIGHT AND DARK: Initial observations of plant response to 2-chloroethyl trimethylammonium chloride revealed that growth was suppressed over a wide range of light intensities. Vegetative extension in tomato seedlings after applying a 10^{-3} M soil drench, was reduced by approximately the same percentage when exposed 12 hours daily to 500, 1,500, and 3,000 ft-c of illuminance. In subsequent studies no growth suppression was observed from 10^{-3} M solutions applied as a soil drench to wheat, squash, bean, and pea seedlings grown in the dark. Vegetative extension was, however, greatly reduced by similar treatment of the same species grown in the light. Results from studies when higher concentrations of the 2-chloroethyl trimethylammonium chloride

TABLE III

EFFECTS OF GIBBERELLIN AND 2-BROMOETHYL TRIMETHYLAMMONIUM BROMIDE SEPARATELY AND IN COMBINATION ON GROWTH OF MUTANT DWARF-1 MAIZE SEEDLINGS

TREATMENTS		LENGTH OF 2ND LEAF SHEATH (MM)	
CHEMICAL	MOLAR	TEST I	TEST II
Control (no treatment)		10	11
Gibberellin	10^{-5}	10	14
	10^{-4}	24	22
2-Bromoethyl trimethylammonium bromide	10^{-4}	...	8
	10^{-3}	3	7
	10^{-2}	2	4
Gibberellin, 2-bromoethyltrimethylammonium bromide	10^{-5}, 10^{-4}	...	17
	10^{-4}, 10^{-4}	...	29
	10^{-5}, 10^{-3}	8	14
	10^{-4}, 10^{-3}	25	19
	10^{-5}, 10^{-2}	17	13
	10^{-4}, 10^{-2}	18	21
Least differences necessary for significance:			
	P=0.05	7	11
	0.01	10	15

and 2-chloroallyl trimethylammonium chloride were applied to bean and pea seedlings grown in light and dark are presented in table IV. There was an especially marked suppression in growth of all bean plants treated with 10^{-2} or 2×10^{-2} M 2-chloroallyl trimethylammonium chloride. Vegetative extension in the dark, however, was not reduced to that observed in the light. Thus in general a greater effect was observed in the light than in the dark. However, with Little Marvel dwarf peas in the light only a slight reduction in height was evident, but heights of those in the dark were greatly suppressed. In other experiments the promotive effects of gibberellin on stem elongation of Little Marvel peas in the dark were antagonized by 2-chloroallyl trimethylammonium chloride.

There appear to be two parameters typical of the effects of these compounds on stem elongation in light and dark. In the light, low concentrations (10^{-4} to 10^{-6} M) reduced vegetative extension of a sensitive plant such as wheat, but there was no effect in the dark. Higher concentrations (10^{-2} and 10^{-3} M) were moderately effective in the dark on all plants tested. The response in the light was manifested by shortened internodes while in the dark the suppression was on hypocotyl elongation. Even in the light, hypocotyl retardation required high concentrations of these chemicals. The response of plants to these chemicals was not an obligated light or dark phenomenon, and varied with plant species. This has also been demonstrated for gibberellin (8).

TABLE IV

EFFECTS OF 2-CHLOROETHYL TRIMETHYLAMMONIUM CHLORIDE AND 2-CHLOROALLYL TRIMETHYLAMMONIUM CHLORIDE ON GROWTH OF PEAS AND BEANS IN LIGHT AND DARK

TREATMENTS		BEANS (*Phaseolus vulgaris*, VAR., CONTENDER)		PEAS (*Pisum sativum*, VAR., LITTLE MARVEL)	
CHEMICAL	MOLAR	LIGHT	DARK	LIGHT	DARK
		Height in cm			
Control (water only)	...	21	30	4.7	21
2-Chloroethyl trimethylammonium chloride	10^{-3}	17	36	3.9	21
	10^{-2}	11	26	4.1	18
	2×10^{-2}	6	18	3.9	16
2-Chloroallyl trimethylammonium chloride	10^{-3}	17	33	4.6	17
	10^{-2}	10	19	3.9	14
	2×10^{-2}	2	10	3.8	12
Least differences necessary for significance:					
	P=0.05	3	4	0.5	2
	0.01	5	6	0.7	3

TABLE V

EFFECTS OF GIBBERELLIN AND ALLYL TRIMETHYLAMMONIUM CHLORIDE ON FLOWERING OF A FACULTATIVE LONG DAY PLANT, LACTUCA SATIVA, VARIETY, GRAND RAPIDS

TREATMENTS		DAYS TO APPEARANCE OF FLOWER PRIMORDIA		HEIGHTS OF FLOWER STALKS (CM)	
CHEMICAL	MOLAR	LONG DAY	SHORT DAY	LONG DAY	SHORT DAY
		(*18 hour*)	(*9 hour*)	(*18 hour*)	(*9 hour*)
Control	...	137	152	66	27
Gibberellin	10^{-4}	131	143	146	58
	10^{-5}	134	146	108	31
Allyl trimethylammonium chloride	10^{-2}	145	158	21	20
	10^{-3}	143	156	28	21
	10^{-4}	140	158	42	22
Gibberellin, allyl trimethylammonium chloride	10^{-4}, 10^{-3}	134	144	108	44
	10^{-5}, 10^{-3}	138	150	52	30
Least differences necessary for significance:	P=0.05	4	2	32	7
	0.01	5	3	42	9

PHOTOPERIODICALLY CONTROLLED FLOWERING RESPONSES: The opposite effects of gibberellin and 2-chloroethyl trimethylammonium chloride and related compounds should be manifest in flowering responses of long and short day plants. Grand Rapids lettuce, a typical facultative long day plant was grown at 9 and 18 hour photoperiods and subjected to the chemical treatments listed in table V. A slight hastening of flowering and the expected increase in flower stalk elongation under both photoperiods occurred with gibberellin. Conversely, allyl trimethylammonium chloride slightly delayed flowering and markedly suppressed the development of the flower stalks. The reduction in height was most pronounced with plants grown under an 18 hour photoperiod and with 10^{-2} M solutions. When the two chemicals were combined intermediate responses occurred. Plants treated with gibberellin at 10^{-5} M and allyl trimethylammonium chloride at 10^{-3} M flowered at approximately the same time as those receiving no chemical treatment. The flower stalks likewise attained about the same height.

Biloxi soybeans exposed to 9 and 18 hour photoperiods flowered, according to expectation, under the short photoperiod and remained vegetative under a long photoperiod. Although pronounced effects on vegetative extension were induced, neither gibberellin nor 2-chloroethyl trimethylammonium chloride altered the flowering behavior. Growth was suppressed in all plants treated with 10^{-2} to 10^{-3} M 2-chloroallyl trimethylammonium chloride, but the effects were most pronounced under long days. Plants treated with 10^{-2} M solutions and grown under long days were vegetative duplicates of nontreated plants exposed to short days. Thus, the equivalent of short day photoperiodic inhibition (11) of vegetative extension was achieved by chemical treatment of a short day plant grown under long days. Flower induction, however, was not achieved.

GROWTH OF TOMATO OVARIES: The relative biological activities of many chemical agents can be readily assessed by measuring their effects on tomato ovary development. A synergistic effect of auxin (IAA) and gibberellin was found for the stimulation of parthenocarpic fruit growth when IAA at 10^{-2} or 10^{-3} M was combined with a 10^{-6} M concentration of gibberellin. However, a higher concentration of gibberellin (10^{-5} M) was as effective by itself as in combination with IAA. This IAA-gibberellin synergism was first reported by Luckwill (10) and is confirmed by the data in table VI.

2-Chloroethyl trimethylammonium chloride used alone had no measurable effect on the growth of nonpollinated tomato ovaries. Similarly, in combination with either gibberellin or IAA there was no consistent response pattern. However, 10^{-2}, 10^{-3}, or 10^{-4} M solutions of 2-chloroethyl trimethylammonium chloride in combination with both 10^{-3} M IAA and 10^{-5} M gibberellin, markedly accelerated ovary growth. This growth was significantly greater than that for any IAA-gibberellin combination or for 4-chlorophenoxyacetic acid at 10^{-4} M. The same concentrations of 2-chloroethyl trimethylammonium chloride used in combination with 10^{-2} M IAA and 10^{-5} M gibberellin caused marked enlargements of the receptacle and peduncle. Ovaries and adjacent tissues appeared as if treated with an excessive application of growth substance. These data suggest that in addition to IAA and gibberellin, possibly a third factor may be involved in the initial stimulus of fruit growth.

DISCUSSION

Visual observations of a wide variety of plant responses relative to the effects of 2-chloroethyl trimethylammonium chloride and gibberellin used singly, in combination, or alternately, suggest that the growth of the plant is controlled by the combined action of

TABLE VI

EFFECT OF 3-INDOLEACETIC ACID, GIBBERELLIN AND 2-CHLOROETHYL TRIMETHYLAMMONIUM CHLORIDE ON GROWTH OF TOMATO OVARIES*

3-INDOLEACETIC ACID MOLAR	GIBBERELLIN MOLAR	2-CHLOROETHYL TRIMETHYLAMMONIUM CHLORIDE MOLAR			
		0	10^{-4}	10^{-3}	10^{-2}
0	0	3.0	3.0	3.0	3.0
	10^{-6}	4.8	9.5	8.3	9.3
	10^{-5}	15.5	16.5	11.8	16.0
	10^{-4}	16.3	19.3	14.5	14.5
10^{-3}	0	4.2	4.0	4.3	3.0
	10^{-6}	12.3	...	...	...
	10^{-5}	16.3	21.7	24.3	26.3
	10^{-4}	16.8	...	...	...
10^{-2}	0	8.2	12.0	11.7	11.3
	10^{-6}	19.2	...	...	...
	10^{-5}	15.3	5.8	15.0	16.8
	10^{-4}	19.0	...	...	...
4-Chlorophenoxyacetic acid					
10^{-4}	0	17.5	...	...	...

* Diameters in millimeters 12 days after treatment.
Least differences necessary for significance between any two values: 0.05 = 6.7, 0.01 = 8.7.

both of these types of compounds. This was first demonstrated for wheat (16) and the tomato (17). Further studies with a variety of biological systems including lettuce seed germination, elongation of Avena coleoptile sections, vegetative extension in normal and dwarf plants, growth of intact plants in light and dark, and photoperiodically controlled flowering responses, tend to confirm that the two classes of compounds induce opposite growth responses. The one exception revealed by the present studies was in parthenocarpic fruit development of the tomato. Here, there was no suppression with 10^{-2} to 10^{-4} M solutions of 2-chloroethyl trimethylammonium chloride of responses from gibberellin (10^{-5} M) or IAA (10^{-3} M) applied separately, but a marked enhancement of the growth induced by the gibberellin and IAA combination. Studies of these and still other combinations of the three groups of growth substances should be explored with the growth of other fruits.

Two conditions have now been reported in which 2-chloroethyl trimethylammonium chloride and related compounds will stimulate growth. Instances of increased vegetative and dry matter accumulation with use of low levels of 2-chloroethyl trimethylammonium chloride and related compounds have been observed (17), where presumably auxin and gibberellin were not limiting. The second condition is the synergistic response from a combination of IAA, gibberellin, and 2-chloroethyl trimethylammonium chloride on parthenocarpic fruit development.

Aside from the promotion of fruit growth the contrasting effects of the two groups of chemicals on plant behavior are apparent with many significant vegetative and reproductive responses. Gibberellin appears to simulate, in many plant species, the effects of a long photoperiod, of exposure to light with energy primarily in the red portion of the visible spectrum, and exposure to high temperatures. Conversely, 2-cholorethyl trimethylammonium chloride and related compounds tend to duplicate the effects of a short photoperiod, combined with a high light intensity of energy primarily in the blue portion of the visible spectrum, and exposure to low temperatures.

No qualitative differences have been detected among the many active compounds of the structure $CH_2X\text{-}CH_2\text{-}N^+(CH_3)_3 \cdot Cl^-$, although marked quantitative differences have been observed with some of the biological systems used. At a given molar concentration the suppression of elongation of pea and bean seedlings in the dark was far more pronounced with 2-chloroallyl trimethylammonium chloride than with 2-chloroethyl trimethylammonium chloride or any other related compounds thus far tested. The vegetative growth inhibition was most strikingly induced with 2-chloroallyl trimethylammonium chloride. This simulated the effects of short days when soybean plants were grown under long days. The effect, however, was strictly quantitative and was easily duplicated by increasing the dosages of a great number of other related compounds. This response pattern was similar to that already encountered for the various gibberellins which, in turn differ in their quantitative but not qualitative effects, and the quantitative response varies with the biological test used for evaluation (1, 2, 4).

Distinct but perhaps related modes of action must be assigned to the three groups of chemical substances, auxins, gibberellins, and analogs of 2-chloroethyl tri-

methylammonium chloride. The chemical structures are so different that it is difficult to conceive that they would be envolved in precisely the same growth systems. There is no evidence that the new growth substances dealt with here occur as natural hormones in plants. There is also no evidence on the mechanism of action of these compounds. While it has been reported that 2-chloroethyl trimethylammonium chloride is a competitive inhibitor of cholinesterase (3), it cannot be assumed that this property is associated with its effects as a plant growth substance. Detection of this enzyme has not been reported in tissues of higher plants. On the other hand, it has been pointed out (14) that the structural similarity of these compounds to choline suggests a possible function in lipid metabolism, and the specificity of the methyl groups in the ammonium cation a role in methylation reactions.

Summary

Vegetative and reproductive responses induced in plants or plant parts by 2-chloroethyl trimethylammonium chloride or related compounds, gibberellin, and 3-indoleacetic acid indicated that closely related, yet distinct systems, were involved. 2-Chloroethyl trimethylammonium chloride suppressed the gibberellin and light induced germination of lettuce seed, vegetative extension of genetically dwarf and normal plants, elongation of plants in light and dark, and flowering of lettuce, a facultative long day plant. With Biloxi soybeans flowering was not affected, but the chemical induced vegetative growth under long days, which was equivalent to a short-day photoperiodic inhibition. Elongation of Avena coleoptile sections was suppressed both in the presence and absence of 3-indoleacetic acid. The suppression of the 3-indoleacetic acid response was partially overcome by gibberellin. There was no direct effect of 2-chloroethyl trimethylammonium chloride on the growth of tomato ovaries. However, in combination with 10^{-3} M 3-indoleacetic acid and 10^{-5} M gibberellin a synergistic growth rate was induced beyond that which could be ascribed to a 3-indoleacetic acid-gibberellin interaction.

The mutually antagonistic effects of 2-chloroethyl trimethylammonium chloride and gibberellin are discussed in light of their contrasts in simulating different light, temperature and photoperiod regimes. Possible modes of action are suggested.

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COMPETITION BETWEEN CHELATING AGENTS AND ROOTS AS FACTOR AFFECTING ABSORPTION OF IRON AND OTHER IONS BY PLANT SPECIES [1]

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Absorption of iron by plants can be increased by adding a chelating agent to a soil (2, 7, 24). Jacobson (9) found that 5 or 10 ppm of iron supplied as FeEDTA in solution cultures provided adequate iron to the plant at high pH. Other workers (7, 11, 12, 19, 20, 23, 24, 25) have confirmed these observations. Plant responses to chelate applications have been shown to differ between plant species (2) and the effectiveness of chelating agents on specific soils are known to differ (2, 24). There has been some indication (4) that roots and chelating agent compete for the Fe in a growth medium. Plant roots from some plant species are more effective competitors than others.

The purpose of this investigation was to study the competition for iron between chelating agents and roots as a factor affecting the absorption of iron and other ions by plants. The investigation was divided into two parts: A. Competition between chelating agents and B: Competition between roots and chelating agents.

MATERIALS AND METHODS

Four chelating agents were used in this investigation, as follows: ethylenediamine-tetra-acetic acid (EDTA), diethylenetriamine-penta-acetic acid (DTPA), cyclohexanediamine-tetra-acetic acid (CDTA), and ethylene-diamine-dihydroxyphenylacetic acid (EDDHA). All of them have been discussed (1, 5, 10, 14) and used experimentally in agriculture as a means of keeping Fe in an available form for plant growth. Solutions of FeEDTA, FeDTPA, FeCDTA, and FeEDDHA have different stability constants, $\log_{10}K$ = 24.8, 27.9, 29.3 (1), and greater than 30.0 (5), respectively.

COMPETITION BETWEEN CHELATING AGENTS. The capacity of EDDHA to chelate Fe was compared in nutrient solution and in water over the pH range of 3 to 8. NaOH or HCl was used to adjust the pH of the nutrient solutions. Fe at 2×10^{-5} M was added as $FeCl_3$. The nutrient solution was purified and contained 26 ppm Ca, 3.3 ppm Mg, 28 ppm N (26.5 as NO_3 and 1.5 as NH_4), 21 ppm K, 0.1 ppm P, 2.4 ppm S, 0.07 ppm Mn, 0.03 ppm B, 0.02 ppm Zn, 0.006 ppm Cu, and 0.006 ppm Mo. EDDHA at 2×10^{-5} M was added to each treatment. Fe in solution as FeEDDHA was determined by measuring the absorbance of the solutions at 480 mμ. The results are expressed as the molar quantity of Fe in solution as FeEDDHA after equilibration for 30 days.

Effects of other chelating agents on the capacity of EDDHA to chelate Fe were determined over the pH range of 3 to 8. EDTA, DTPA, and CDTA (1.5×10^{-5} M) were equilibrated for 1 hour with 2×10^{-5} M Fe as $FeCl_3$ in nutrient solution. The components were added in the following order: $FeCl_3$, chelating agent, and nutrient solution. EDDHA at 1.5×10^{-5} M was then added to each of the nutrient cultures and FeEDDHA concentration was determined by measuring the absorbance of the solutions at 480 mμ (22). Nutrient solutions and chelating agent were mixed and equilibrated at the respective pH selected. NaOH or HCl was used to adjust the pH. Measurements were made over a period of 30 days. Fifty to 80 % of the FeEDDHA which formed in 30 days was formed in 24 hours. The least amount of FeEDDHA had been formed where the greatest competition existed for Fe. But at all periods of measurement the data were comparative and resulted in the same conclusions. Thirty days were considered sufficient time to allow a fair comparison between chelating agents.

At pH 6.5, the capacity of EDDHA to chelate Fe was determined in nutrient solution containing variable concentrations of competitive chelating agents. EDTA, DTPA, and CDTA were equilibrated for 1 hour with nutrient solution containing 2×10^{-5} M and 4×10^{-5} M Fe as $FeCl_3$. Chelating agent concentration varied from zero to 36×10^{-5} M at each of the two iron levels. After equilibration, 2×10^{-5} M EDDHA was added to each of the nutrient solutions and FeEDDHA concentration was determined by measuring the absorbance of the solutions at 480 mμ. Final measurements were made after 30 days. All of these experiments were repeated three times.

COMPETITION BETWEEN ROOTS AND CHELATING AGENTS. Red kidney beans (*Phaseolus vulgaris* L.), soybeans [*Glycine max* (L.), var. PI-54619-5-1], and sorghum (*Sorghum vulgare* pers., var. Wheatland milo) were used to study the chelating capacity or

[1] Received April 18, 1960.

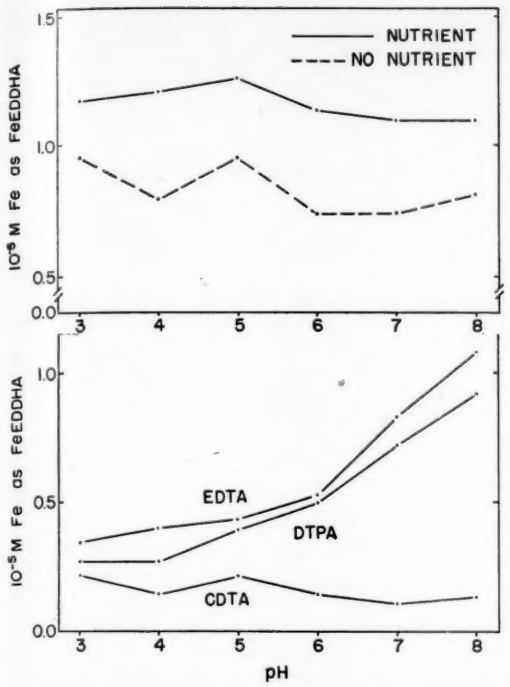

FIG. 1 (*top*). Capacity of EDDHA to form FeEDDHA in nutrient solution and in water over the pH range of 3 to 8. (*bottom*). Capacity of EDDHA (1.5×10^{-5} M) to form FeEDDHA in nutrient solution containing 2×10^{-5} M Fe and 1.5×10^{-5} M EDTA, DTPA, and CDTA over the pH range of 3 to 8. Fe was equilibrated with competitive EDTA, DTPA, and CDTA before EDDHA was added to the solution.

absorption of iron by their roots as affected by variable concentrations of chelating agents in nutrient solutions. The nutrient solution was the same as described previously. EDTA, DTPA, CDTA, and EDDHA were added in all experiments to make 0.16, 1.0, 2.0, 6.0, and 18×10^{-5} M concentrations. While iron concentration was held constant in each experiment, in the overall series of experiments Fe as $FeCl_3$ was successively raised from 1×10^{-5} M to 6×10^{-5} M. Four replications were used. Each treatment was contained in 8 liters of nutrient solution adjusted and maintained at pH 6.5.

Seeds were germinated at a temperature of 75° F for 48 hours between moist muslin placed on stainless steel screens. The young seedlings were transplanted to the nutrient solutions for 3 days to allow root elongation and then transplanted to their respective treatments. Each plant was supported by a cork placed in the top of a lid covering the nutrient solution. Four plants were grown in each treatment. In most cases the plants were harvested as soon as growth differences were evident or chlorosis had developed. Tops and roots were harvested separately and dried at 70° F in a force-draft oven. The roots were washed thoroughly in demineralized water before drying. Dry weight was recorded for both roots and tops.

The plant material was ground in a Wiley mill to pass a 40 mesh sieve. Ca, Mg, K, Na, P, B, Fe, Al, Mn, and Cu were determined spectrochemically (18) in the tops. The concentration of the above ten elements was also determined in the nutrient solution for each treatment at the time the plants were harvested. Fe (17) and Mn (16) were determined in the roots.

RESULTS

COMPETITION BETWEEN CHELATING AGENTS. Over the pH range of 3 to 8, EDDHA in nutrient solution chelated more Fe than EDDHA in water alone (fig 1). Over the same pH range, CDTA competed with EDDHA for Fe in nutrient solution more successfully than DTPA or EDTA (fig 1). EDDHA chelated much less Fe from FeEDTA and FeDTPA at pH 5.0 than at pH 8.0.

Concentration of chelating agent was found to be a factor affecting chelating capacity. EDDHA was used as a standard to measure the effects of concentration of other chelating competitors on chelating capacity of EDDHA. FeEDDHA has the highest stability constant (1, 5) of the chelating agents tested. With the concentration of EDDHA and Fe constant

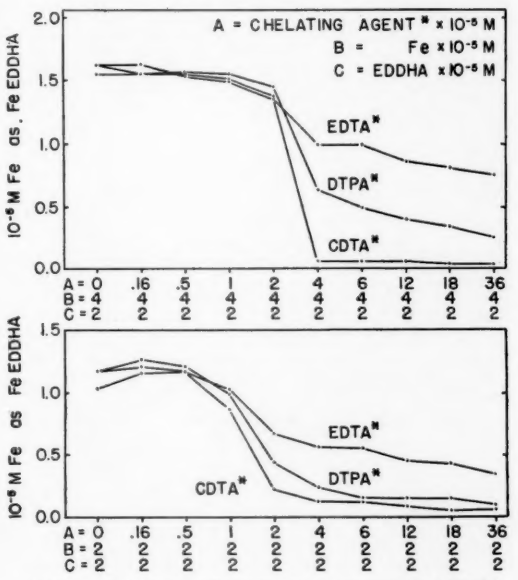
A = CHELATING AGENT* x 10⁻⁵ M
B = Fe x 10⁻⁵ M
C = EDDHA x 10⁻⁵ M
10⁻⁵ M Fe as Fe EDDHA
EDTA*
DTPA*
CDTA*
A = 0 .16 .5 1 2 4 6 12 18 36
B = 4 4 4 4 4 4 4 4 4 4
C = 2 2 2 2 2 2 2 2 2 2
10⁻⁵ M Fe as Fe EDDHA
EDTA*
CDTA*
DTPA*
A = 0 .16 .5 1 2 4 6 12 18 36
B = 2 2 2 2 2 2 2 2 2 2
C = 2 2 2 2 2 2 2 2 2 2

FIG. 2. Effect of concentration of competitive EDTA, DTPA, and CDTA on the capacity of EDDHA to form FeEDDHA at pH 6.5: (*top*) at 4×10^{-5} M Fe and (*bottom*) at 2×10^{-5} M Fe.

at 2×10^{-5} M, chelating capacity of EDDHA (formation of FeEDDHA) remained rather constant until the concentration of the competing chelating agent approached and exceeded the concentration of the Fe (fig 2). At 1×10^{-5} M, EDTA, DTPA, or CDTA chelation by EDDHA decreased; EDTA < DTPA < CDTA (fig 2). The chelating capacity of EDDHA decreased much more sharply at 2×10^{-5} M concentration for each of the chelating agents tested. At this point, 2×10^{-5} M EDTA, DTPA, or CDTA were each competing with 2×10^{-5} M EDDHA for 2×10^{-5} M of Fe (fig 2). By increasing the Fe concentration to 4×10^{-5} M, 2×10^{-5} M EDDHA competed successfully with 2×10^{-5} M EDTA, DTPA, and CDTA for Fe. In this case there was sufficient iron to satisfy the chelating capacity of EDDHA and the particular chelator competing with EDDHA. An increase in concentration of EDTA, DTPA, or CDTA to 4×10^{-5} M sharply decreased the amount of Fe chelated as FeEDDHA. The effectiveness of the competitors was EDTA < DTPA < CDTA (fig 2). Thus, chelating capacity of EDDHA is dependent upon both the concentration of the Fe and the competitive chelating agent in solution.

Competition Between Roots and Chelating Agents. Roots of plants were used as standards, instead of EDDHA, to measure chelating capacity or capacity of roots to absorb iron from nutrient solution. Concentration of chelating agent was again found to be a factor affecting chelating capacity or capacity of roots to absorb iron. With the concentration of Fe constant at 2×10^{-5} M, PI soybeans developed chlorosis at 6×10^{-5} M DTPA and CDTA (fig 3). The plants remained green at 6×10^{-5} M EDDHA and EDTA. Chlorosis did not develop in PI soybeans at 6×10^{-5} M Fe until DTPA and CDTA concentration in nutrient solution was 18×10^{-5} M (fig 3).

Milo plants did not respond like PI soybeans (fig 4). With the concentration of Fe constant at 2×10^{-5} M, milo developed chlorosis when the concentration of EDDHA was 1×10^{-5} M. Chlorosis did not develop in milo at 6×10^{-5} M Fe until the EDDHA concentration was 18×10^{-5} M (fig 4). Milo developed chlorosis when the concentration of EDTA, DTPA, and CDTA was 6×10^{-5} M with Fe constant at 2×10^{-5} M (fig 4). Chlorosis was corrected by adding more Fe to the nutrient solution.

Internode elongation was affected in red kidney beans as the concentration of DTPA increased in nutrient solution (fig 5). With the concentration of Fe constant at 1×10^{-5} M, internode elongation practically stopped at 2×10^{-5} M DTPA. Chlorosis developed in red kidney beans at DTPA concentrations

Fig. 3. PI soybeans grown on complete nutrient solution containing four separate chelating agents. (*top to bottom*). EDDHA, CDTA, DTPA, and EDTA. Plants on left were in nutrient solutions which contained 2×10^{-5} M Fe, on the right 6×10^{-5} M Fe. Concentration of chelating agent was increased for each treatment (*left to right*) as follows: 0.16, 1, 2, 6, and 18×10^{-5} M.

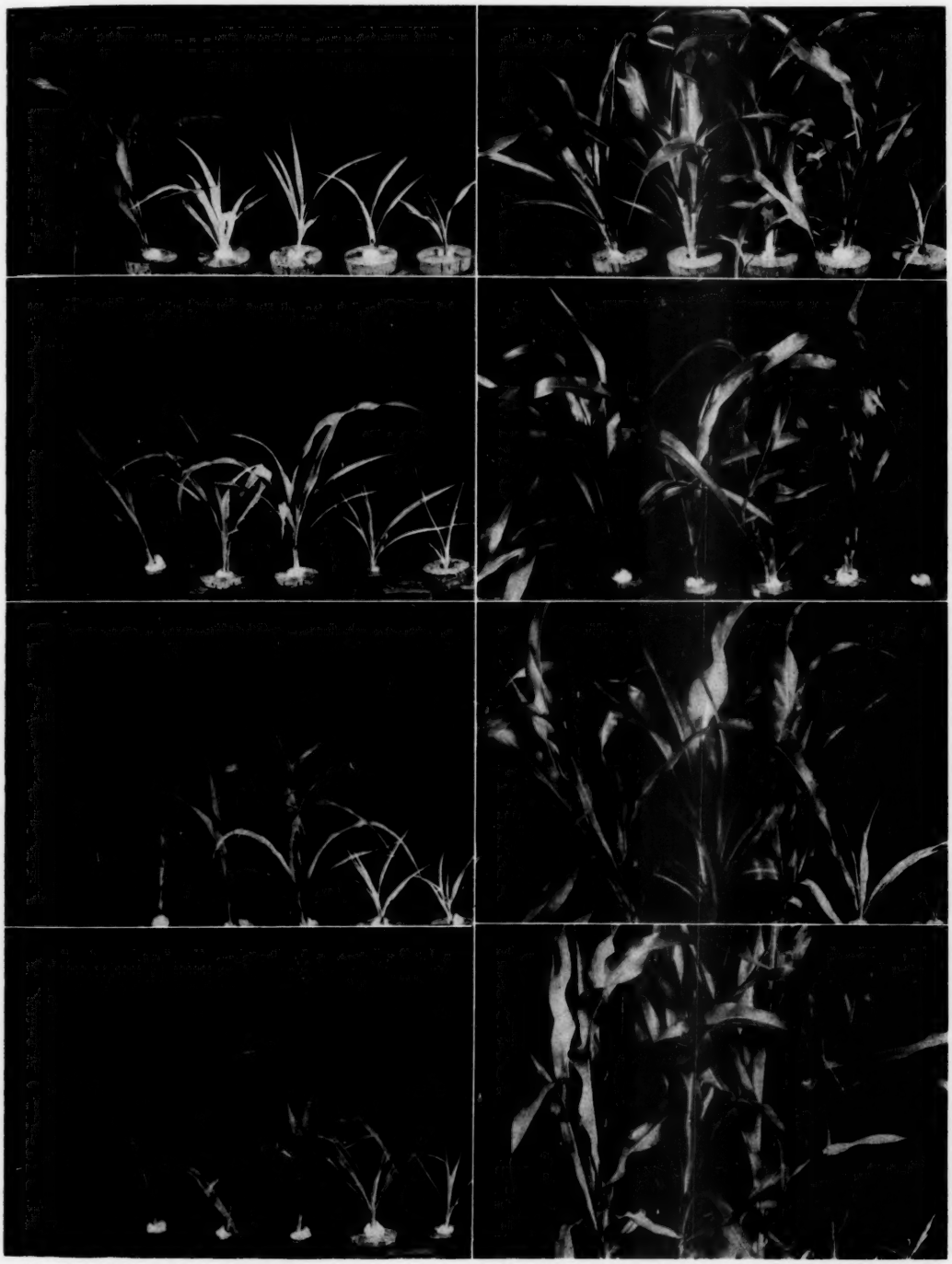

FIG. 4. Milo grown on complete nutrient solution containing four separate chelating agents. (*top to bottom*), EDDHA, CDTA, DTPA, and EDTA. Plants on left were in nutrient solutions which contained 2×10^{-5} M Fe, on the right 6×10^{-5} M Fe. Concentration of chelating agent was increased for each treatment (*left to right*) as follows: 0.16, 1, 2, 6, and 18×10^{-5} M.

TABLE I

ELEMENT CONCENTRATION IN RED KIDNEY BEANS AS AFFECTED BY CONCENTRATION OF CHELATING AGENT IN NUTRIENT SOLUTION CONTAINING Fe AT 1×10^{-5} M

TREATMENTS CHELATING AGENT $\times 10^{-5}$ M		ELEMENT CONCENTRATION—PPM											
		IN PLANT TOP								IN NUTRIENT CULTURE*			
		Ca $\times 10^4$	Mg $\times 10^4$	K $\times 10^4$	P $\times 10^4$	B	Fe	Mn	Cu	Ca	Fe	Mn	Cu $\times 10^{-1}$
EDDHA	0.16	2.2	0.27	1.6	0.24	38	105	23	5.6	ND**	0.02	ND	0.01
	1.00	1.8	0.27	1.6	0.22	34	89	21	4.5	2.1	0.11	ND	0.01
	2.00	2.3	0.28	1.6	0.28	38	110	30	5.1	1.3	0.05	ND	0.01
	6.00	1.8	0.27	1.8	0.24	35	96	31	3.3	1.6	0.09	ND	0.01
	18.00	2.6	0.27	1.8	0.23	35	75	44	3.6	2.4	0.18	0.01	0.04
CDTA	0.16	1.6	0.28	1.4	0.25	39	110	22	6.0	ND	0.02	ND	0.01
	1.00	1.7	0.29	1.7	0.32	39	76	25	4.7	1.9	0.10	ND	0.01
	2.00	2.3	0.29	1.9	0.36	50	73	66	2.5	1.6	0.09	0.01	0.01
	6.00	2.9	0.26	1.8	0.31	49	28	44	2.2	5.6	0.44	0.06	0.08
	18.00	3.1	0.36	3.2	0.46	51	13	13	2.3	10.5	0.70	0.09	0.09
DTPA	0.16	1.5	0.27	1.4	0.21	31	88	22	4.3	1.1	0.02	ND	0.01
	1.00	1.7	0.26	1.5	0.22	35	78	22	3.1	2.3	0.18	ND	0.02
	2.00	3.2	0.22	1.6	0.36	46	83	70	2.0	2.0	0.11	0.01	0.01
	6.00	2.8	0.22	1.5	0.36	51	43	39	2.1	4.3	0.32	0.03	0.06
	18.00	3.1	0.42	3.4	0.52	66	23	33	3.8	13.5	0.55	0.07	0.08
EDTA	0.16	1.6	0.29	1.6	0.25	35	94	20	5.6	1.0	0.02	ND	0.01
	1.00	1.8	0.27	1.6	0.23	34	75	24	3.5	2.1	0.12	ND	0.02
	2.00	2.1	0.30	1.7	0.28	45	103	35	2.6	2.1	0.14	0.01	0.02
	6.00	2.1	0.29	1.9	0.33	47	105	61	2.7	2.6	0.19	0.01	0.05
	18.00	2.2	0.32	2.1	0.38	47	84	43	3.0	6.8	0.46	0.04	0.09

* After containing red kidney beans 10 days.
** Not detectable spectrochemically.

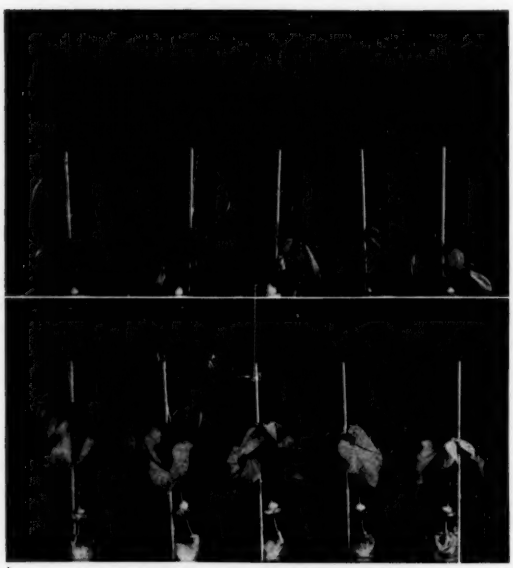

FIG. 5. Red kidney beans grown on complete nutrient solution containing increasing concentrations of DTPA (*left to right*) as follows: 0.16, 1, 2, 6, and 18×10^{-5} M. Plants at top received 2×10^{-5} M Fe; plants at bottom received 1×10^{-5} M Fe in each treatment. Note (*bottom*) failure of internodes to elongate at 2×10^{-5} M DTPA.

of 18×10^{-5} M. When the Fe concentration was increased to 2×10^{-5} M, some internode elongation occurred at 2×10^{-5} M DTPA and chlorosis did not develop at 18×10^{-5} M DTPA (fig 5).

Total Fe in the tops of red kidney beans was affected by the chelating agent concentration in the nutrient solution (fig 6). With the concentration of Fe constant at 1×10^{-5} M, total iron concentration in the plant decreased with increasing concentration (0.16×10^{-5} M to 1×10^{-5} M) of EDDHA, CDTA, DTPA, and EDTA in nutrient solution. With further increase in chelating agent (2×10^{-5} M), total iron increased in plants from EDDHA and EDTA treatments while in DTPA and CDTA treatments there was a marked decrease in total iron (fig 6). Total iron in plant tops decreased further with greater concentrations of chelating agent in the

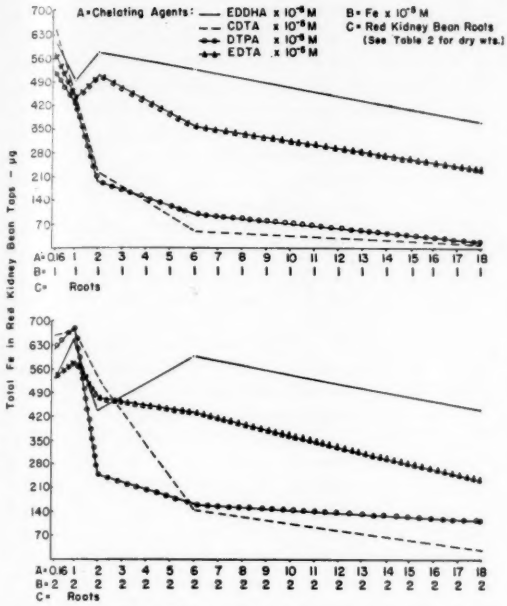
A=Chelating Agents: EDDHA x 10⁻⁵ M
CDTA x 10⁻⁵ M
DTPA x 10⁻⁵ M
EDTA x 10⁻⁵ M
B= Fe x 10⁻⁵ M
C= Red Kidney Bean Roots
(See Table 2 for dry wts.)
Total Fe in Red Kidney Bean Tops - γg
A=0.16 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B= 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
C= Roots
A=0.16 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B=2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
C= Roots

FIG. 6. Effect of increasing the concentration of four chelating agents in nutrient solution on the total iron absorbed by red kidney beans at two Fe levels: (*top*) 1×10^{-5} M Fe and (*bottom*) 2×10^{-5} M Fe. Note the difference in the slope of the curves at the two Fe levels with 1×10^{-5} M chelating agent in solution.

nutrient solution: CDTA > DTPA > EDTA > EDDHA.

When the concentration of Fe was increased to 2×10^{-5} M, total Fe absorbed by red kidney beans increased on treatments containing 1×10^{-5} M chelating agent. Further increase in the concentration of the competing chelators decreased the absorption of Fe by the plants (fig 6).

Element concentration in red kidney beans was affected by the concentration of chelating agent in the nutrient solution (tables I & II). As the concentration of chelating agent increased, Fe and Cu concentration in the tops of the plants decreased. Iron decreased from 110 ppm in the 0.16×10^{-5} M CDTA treatment to 13 ppm in the 18×10^{-5} M CDTA treatment. Ca, P, and B concentration increased most in the plant tops containing the least amount of Fe. More Fe, Ca, Mn, and Cu were held in solution (not absorbed by the root) in nutrient solutions containing the largest quantity of chelating agent (table I).

TABLE II

ELEMENT CONCENTRATION IN RED KIDNEY BEANS AS AFFECTED BY CONCENTRATION OF CHELATING AGENT IN NUTRIENT SOLUTION CONTAINING Fe AT 2×10^{-5} M

TREATMENTS CHELATING AGENT $\times 10^{-5}$ M		ELEMENT CONCENTRATION—PPM											
		IN PLANT TOP								IN NUTRIENT CULTURE*			
		Ca $\times 10^4$	Mg $\times 10^4$	K $\times 10^4$	P $\times 10^4$	B	Fe	Mn	Cu	Ca	Fe	Mn	Cu $\times 10^{-1}$
EDDHA	0.16	3.2	0.27	1.8	0.29	40	120	30	6.5	ND**	0.03	ND	0.01
	1.00	2.4	0.30	1.8	0.35	38	130	31	6.5	2.4	0.21	ND	0.02
	2.00	2.8	0.26	1.8	0.32	39	109	37	6.5	3.6	0.38	ND	0.01
	6.00	3.4	0.28	1.8	0.35	38	148	53	3.7	3.9	0.44	ND	0.02
	18.00	3.1	0.27	1.8	0.29	32	108	66	2.6	3.5	0.34	0.01	0.04
CDTA	0.16	2.0	0.29	1.7	0.30	32	126	31	4.7	1.4	0.02	ND	0.01
	1.00	2.4	0.29	1.9	0.28	32	135	30	4.6	2.3	0.18	ND	0.02
	2.00	2.0	0.28	1.7	0.29	36	109	24	3.6	2.4	0.20	ND	0.01
	6.00	3.2	0.27	1.7	0.42	42	68	35	2.0	12.8	0.98	0.07	0.05
	18.00	3.5	0.45	3.3	0.55	49	38	19	2.3	21.6	1.18	0.09	0.07
DTPA	0.16	1.9	0.26	1.5	0.24	30	121	26	5.3	1.0	0.02	ND	0.01
	1.00	2.4	0.24	1.7	0.27	32	138	34	6.3	2.5	0.21	ND	0.04
	2.00	3.0	0.22	1.8	0.32	34	74	40	2.1	6.4	0.58	0.03	0.07
	6.00	2.6	0.23	1.3	0.36	41	68	34	2.2	13.7	1.08	0.06	0.07
	18.00	2.5	0.33	2.2	0.44	40	71	35	2.3	18.5	1.20	0.07	0.08
EDTA	0.16	2.7	0.26	1.9	0.33	39	118	29	7.2	ND	0.02	ND	0.01
	1.00	2.6	0.27	2.1	0.24	32	133	33	4.8	1.2	0.03	ND	0.01
	2.00	2.9	0.24	1.9	0.33	39	110	32	4.5	2.0	0.18	ND	0.01
	6.00	3.0	0.25	1.9	0.42	39	133	39	2.6	4.4	0.43	0.02	0.05
	18.00	2.2	0.25	2.1	0.41	42	99	33	2.2	11.8	1.00	0.05	0.07

* After containing red kidney beans 10 days.
** Not detectable spectrochemically.

Roots and chelating agents all competed for the iron in the growth medium. As the concentration of chelating agent increased, iron was withheld from the plant and iron chlorosis developed. Chlorosis was corrected (figs 3, 4, 5) and the yields increased (table 3), by adding more iron to the nutrient solutions. Fe and Mn concentration in the roots followed the same trend as that found in the tops of the plants.

Discussion

This investigation has shown that chelating agents can compete with plant roots for Fe and that plant species differ in their capacity to absorb Fe from metal chelates. Preliminary experiments were done to characterize the four chelating agents which were used in the investigation. It was found that EDDHA chelated Fe much better in nutrient solution than in water. Most of the Fe in water probably existed as $Fe(OH)_3$, making it less available for chelation than the forms of Fe which would exist in the solution culture. The capacity of EDDHA to chelate Fe was measured with EDTA, DTPA, and CDTA in the solution culture as competing chelating agents. EDDHA had the capacity to form more FeEDDHA with EDTA as the competing chelating agent than with either DTPA or CDTA as competitors. They ranked in the order of their stability constants in competing with EDDHA for Fe: EDTA < DTPA < CDTA.

Our next objective was to determine how competitive EDTA, DTPA, and CDTA would be if Fe and EDDHA concentrations were kept constant and the other chelating agents added at increasing concentrations. When chelating agents and Fe were in equal molar concentrations, EDDHA competed successfully with EDTA, DTPA, and CDTA for Fe. But with a further increase in the concentration of EDTA, DTPA, and CDTA, these chelating agents competed with EDDHA for Fe in the order: EDTA < DTPA < CDTA. More EDTA, DTPA, and CDTA were required to be competitive if the Fe concentration in the solution culture was increased. From the stability constants given for the chelating agents one would not expect to find this competition. FeEDDHA has a stability constant at least 10,000 times greater than that of FeEDTA; if other things were equal, EDTA would not compete observably with EDDHA for Fe until present in large excess. Competition was observed in these studies when the EDTA concentration was doubled. We do not feel qualified to try to explain the chemical behavior of the chelate systems at this time, but we do feel there is real significance in the observation of chelating agent competition for Fe (possibly other elements) in plant metabolism.

Table III

Effect of Concentration of Chelating Agents in Nutrient Solution at Variable Iron Levels on Yield of Red Kidney Beans, PI Soybeans, and Milo

Treatments			Yield (dry wt)		Treatment	Yield (dry wt)			Treatment	Yield (dry wt)	
Chelating agents $\times 10^{-5}$ M		Fe $\times 10^{-5}$ M	R.K.B.* Tops	R.K.B.* Roots	Fe $\times 10^{-5}$ M	R.K.B.	PI** Tops	Milo	Fe $\times 10^{-3}$ M	PI Tops	Milo Tops
			g	g		g	g	g		g	g
EDDHA	0.16	1	5.8	2.4	2	4.4	1.2	1.7	6	1.2	1.7
	1.00	1	5.5	2.2	2	5.0	1.3	0.4	6	1.0	4.6
	2.00	1	5.3	2.5	2	4.0	1.4	0.2	6	1.3	4.8
	6.00	1	5.5	2.3	2	4.1	1.2	0.2	6	1.0	4.1
	18.00	1	5.5	2.4	2	4.1	1.2	0.1	6	0.9	0.2
CDTA	0.16	1	5.9	2.5	2	5.2	1.5	1.1	6	1.1	7.9
	1.00	1	6.3	2.4	2	5.0	1.3	1.1	6	1.3	6.8
	2.00	1	3.1	3.1	2	5.0	1.5	1.2	6	1.2	4.9
	6.00	1	2.0	3.0	2	2.1	0.4	0.3	6	1.5	5.9
	18.00	1	1.0	2.1	2	0.8	0.4	0.1	6	0.5	0.1
DTPA	0.16	1	6.0	2.4	2	5.1	1.4	1.8	6	1.0	8.5
	1.00	1	5.5	2.7	2	5.0	1.4	1.7	6	1.1	8.3
	2.00	1	2.4	3.2	2	3.3	1.1	1.0	6	0.9	8.9
	6.00	1	2.3	3.4	2	2.4	0.7	0.2	6	1.0	0.5
	18.00	1	0.8	1.8	2	1.6	0.4	0.1	6	0.6	0.1
EDTA	0.16	1	6.1	2.3	2	4.6	1.1	2.6	6	1.0	11.0
	1.00	1	5.9	2.4	2	4.4	1.2	1.7	6	1.0	8.9
	2.00	1	5.0	2.5	2	4.3	1.2	1.5	6	1.2	11.5
	6.00	1	3.4	2.8	2	3.3	1.0	0.4	6	1.1	8.3
	18.00	1	2.8	2.9	2	2.4	0.9	0.2	6	0.9	0.1

* Red kidney beans.
** PI-54619-5-1 soybeans.

Chelating agents are numerous in plants and the kind and concentration are dependent upon the plant species.

In the second phase of this investigation, our objective was to determine if there is competition between chelating agents and roots for Fe. Roots, in these experiments are analogous to EDDHA in the competitive experiments using chelating agents in vitro. Roots were found to differ, just as chelating agents differ, in their capacity to compete for Fe. Wheatland milo was unable to absorb and utilize Fe from FeEDDHA unless the Fe concentration exceeded the EDDHA concentration. In contrast, PI soybeans and red kidney beans had the capacity to absorb Fe from FeEDDHA when the EDDHA concentration was nine times greater than the Fe concentration. A probable explanation is that in some way the stability of the metal chelate is changed at the root by the beans. This could be done at the root by changing $Fe^{+++} \rightarrow Fe^{++}$ (3). Bond and Jones (1) have shown that the stability constants of some ferrochelates are less than the ferrichelates. Reductive capacity at the root may be a factor related to Fe absorption and analogous to the observations which have been made concerning Fe absorption in animals. Loewus and Fineberg (13) suggest that the storage and mobilization of Fe in animals is controlled by its oxidation and reduction. In ferritin, the normal mammalian iron storage protein, the iron exists in the ferric state and is released from the protein only after reduction to the ferrous state. Granick (6) found that plasma iron in transport to storage depots occurs in the ferric state. Its storage presumably requires an intermediate reduction. Like the Fe^{+++} in the animal, Fe^{+++} at the root may be a rather immobile form of Fe. Ferrous iron may be more mobile in that the ferrous-chelate is less stable and the roots can compete more effectively for it.

Where Fe is held from the plant root by the metal chelate, Mn, Ca, Mg, B, P, and K continue to be absorbed. Some of these ions could act competitively against the proper functioning of Fe if their concentration were allowed to increase. Taper and Leach (21) and Olsen (14a) have found that the absorption of Fe by plants can be decreased by increasing the concentration of Ca in the solution culture. Hunter and Vergnano (8) found the effectiveness of some microelements to produce iron chlorosis in oats to be in the following order: Ni > Cu > Co > Cr > Zn > Mo > Mn. Reuther and Smith (15), using sand culture, demonstrated that an excess of copper caused iron chlorosis in citrus. Thus, it appears that absorption of Fe by roots may not only be affected by the concentration of a chelating agent in solution, but may be competitively affected by the concentration of other micro and macroelements in solution.

Just as concentration of a competing metal ion may affect the available or active iron in a plant, plant species may have the capacity to alter the activity of a metal ion by increasing or decreasing the concentration of a specific chelating agent inside the plant or in root exudate. In this way, kind and concentration of a chelating agent coupled with iron supply in a growth medium are important factors affecting the absorption of iron and probably other nutrient elements by plants.

Summary

Plant species differ in their capacity to absorb iron from chelating agents. In vitro experiments demonstrated a competition between chelating agents for iron. In vivo experiments showed that roots react like chelating agents in their capacity to compete for the iron in a growth medium. As the molar concentration of chelating agent was increased to exceed that of iron, the amount of iron absorbed by the plant decreased. The competitive effect of chelating agent was overcome by adding more iron to the nutrient solution. The concentration of iron and chelating agent in a growth medium are discussed as controlling factors affecting the absorption of iron and other nutrient elements by plants.

Acknowledgment

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BIOSYNTHESIS OF ANABASINE AND OF NICOTINE BY EXCISED ROOT CULTURES OF NICOTIANA GLAUCA [1, 2, 3]

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Investigations of Nicotiana alkaloid biogenesis have tended thus far to center about nicotine as the archetype of the group. Sufficient progress has been made to justify extending both the methods and the concepts of these investigations to other members of the alkaloid complex. The case of anabasine is especially attractive for the following reasons.

First, anabasine is a minor alkaloid accompanying nicotine in *Nicotiana tabacum* L. However, it is the major alkaloid in *N. glauca* Grah. where it is accompanied by moderate amounts of nicotine. The latter species thus presents opportunity for investigating the formation of both substances in one and the same system using the known characteristics of nicotine production as convenient internal points of reference.

Second, it was shown earlier (6) that relative anabasine and nicotine producing capacities are not shared equally by all portions of the plant body. Extension of this aspect of the study may be expected to reveal the degree of separation and perhaps also to delimit the compartment associated with the production of each alkaloid.

Third, the excised root system of *N. glauca* would appear to afford an ideal experimental tool for investigating the rate stability of alkaloid production. Elsewhere, we report the fact (9) that supplying precursors of nicotine to excised root cultures of *N. tabacum* does not increase nicotine yields. The present system might be used to learn whether nicotine production could be diverted to anabasine production, or vice versa, by supplying the corresponding precursor in excess.

MATERIALS AND METHODS

EXCISED ROOT CULTURES: A clonal line of *Nicotiana glauca* Grah. was established from one sterile seedling. White's medium (18) was employed with the customary additions of copper and molybdenum (16). The behavior of this clone in sterile culture is worth noting.

In the first passages, the clone exhibited pronounced dominance of the primary root such that branching of the second and higher orders was curtailed. The primary root grew rapidly and traversed the periphery of the culture flask (125 ml Erlenmyer) many times to form a coiled arrangement. Cultures remaining in this state were designated subclone A. After more than a year of culture, however, the dominance of the primary root diminished, and root branches of secondary and higher orders became common. In the latter condition, the cultures were similar in appearance to the excised root cultures of *N. tabacum* L. var. Turkish which have been grown for many years in this laboratory. Such cultures were isolated and maintained through subsequent passages as subclone B. The relation between root morphology and alkaloid production rates is unknown. The data recorded herein have been obtained with the use of subclone B except where otherwise indicated. For more than a year following initial isolation of the clone, the cultures exhibited a strong tendency to regenerate shoot buds.

GROWTH AND ALKALOID PRODUCTION: For measuring growth and alkaloid production rates, suitable numbers of cultures were prepared, incubated at 30 $\pm$ 1° C, and harvested in lots of 50 each at stated intervals. Such measurements were made in two separate experiments.

ADDING INTERMEDIATES: In general, new cultures in lots of 50 to 100 were prepared and incubated for 1 week prior to adding intermediates. The intermediates were filter sterilized through Pyrex fritted glass disks (UF) or autoclaved depending upon compound lability. Incubation was continued for an additional 4 weeks after adding the intermediates.

Nonlabeled intermediates were supplied to observe the effects upon relative yields of anabasine and nicotine. In one series, DL-ornithine and L-lysine were supplied separately at the level of 1.0 mg per culture to duplicated lots; controls without added precursor were provided in quadruplicate. In a second series, lysine and ornithine were again supplied as above but with additions of 0.3 mg of nicotinic acid per culture. Figures for the effects of supplying nicotinic acid alone were secured in triplicate from other experiments in which the alkaloids were separated and assayed gravimetrically as the dipicrates. Ratios of anabasine to nicotine yields were calculated, and the resulting figures, despite their heterogeneous origins, were subjected to an analysis of variance using a 2×3 factorial design with the usual modifications for unequal lot numbers.

For measuring the available nicotinic acid in the root cultures during the exponential period of growth, two experiments were completed in each of which five lots of 20 cultures each were harvested on the 11th, 14th, 17th, and 20th days, respectively, of the passage.

[1] Received April 18, 1960.

[2] Work completed in part under the auspices of the United States Atomic Energy Commission at Columbia University under contract AT-30-1-1778 and at Brookhaven National Laboratory and of the Tobacco Industry Research Committee.

[3] The work on nicotinic acid recovery was performed by Miss Dorothy Hufnagel, Tobacco Industry Research Committee Fellow in Botany at Columbia, 1957 to 1959.

Total acid-hydrolyzable nicotinic acid content of the roots was measured by a bioassay procedure using *Lactobacillus plantarum* (Orla-Jensen) Holland, strain 17-5, as test organism (2). In another experiment, 337 μg of nicotinic acid were added to each of 20 cultures and the amount remaining on the 20th day ascertained by assay.

LABELED INTERMEDIATES: L-Lysine-C^{14} (2.3×10^7 dpm/mg) was purchased from Schwarz Laboratories, Inc. DL-Ornithine-2-C^{14} monohydrochloride (8.2×10^6 dpm/mg) was purchased from Tracerlab. Nicotinic acid labeled with tritium in position 2 was prepared from 2-bromo-3-picoline by methods described elsewhere (9). Two preparations were employed with specific activities 2.25×10^5 and 2.57×10^5 dpm/mg, respectively. Nicotinic acid ring-labeled with tritium was also prepared by the reaction Li^6 (n, α)H^3 in the Brookhaven reactor (1, 21, 22). The specific activity of the latter after purification and dilution (9) was 2.65×10^5 dpm/mg. 6-Tritium nicotinic acid was prepared from 6-bromo-3-picoline (9) with specific activity 2.16×10^5 dpm/mg.

Circumstances surrounding the addition of these compounds to the culture medium were similar to those already described in a preceding section except for such details as are indicated in table I.

ISOLATION AND SEPARATION OF ALKALOIDS: The roots were removed from the culture flasks and washed with distilled water on a Buchner funnel. The washings were combined with the spent culture fluids. The roots were ground with a little trichloroacetic acid and then extracted twice with boiling water. The filtered aqueous extracts were combined with the spent culture fluids and washings and the volume reduced at lowered pressure. The alkaloids were extracted from the alkalinized concentrate continuously with ether for 72 hours.

The ethereal extracts were concentrated to low volume and transferred to columns packed under buffer-saturated ether with Johns-Manville Hyflo-SuperCel moistened (0.2 ml/g) with the same 0.5 M potassium phosphate buffer (pH 6.6) (7). Ether saturated with buffer was passed through this column under pressure and fractions collected automatically. When the nicotine fraction had left the column, the solvent was changed to ether-chloroform (1:1) and anabasine was eluted. In certain cases, when the anabasine was just off the column, the solvent was changed to pure chloroform and nornicotine eluted in the next two collections.

For assay, the different collections were combined according to alkaloid composition and transferred to N HCl in a separatory funnel. The nicotine fraction was steam-distilled onto an Amberlite IRC-50 resin cartridge and eluted with HCl to remove nonvolatile impurities (7). Anabasine and nornicotine fractions did not require distillation for purposes of assay. The amounts of each alkaloid were estimated spectrophotometrically (19) using physical constants established in this laboratory.

When the alkaloids were to be isolated as picrates, the fractions were steam distilled into aqueous picric acid solution (nicotine from excess MgO and anabasine from excess NaOH and NaCl). The distillates were taken to low volume under reduced pressure, and the picrates dissolved by boiling with the least amount of water and then allowed to crystallize at room temperature. Solubility corrections were applied when calculating picrate recoveries for estimating radiochemical yields. In still other cases, the initial ethereal extracts were applied directly to filter paper sheets (Whatman No. 1) for chromatography.

ISOTOPE ANALYSIS: Samples of the alkaloid picrates were assayed for C^{14} and for tritium by combustion and proportional gas counting (3, 4, 5, 20). Results are expressed as radiochemical yield, that is, the proportion of radioactivity originally supplied to the cultures which was recovered in a given alkaloid fraction. Specific activities of tritium-labeled compounds were corrected for isotope decay.

PAPER CHROMATOGRAPHY OF ALKALOIDS: Ethereal solutions of the alkaloids were concentrated to low volume and applied to sheets of Whatman No. 1 filter paper which had been impregnated earlier with 0.2 M acetate buffer (pH 5.6). The chromatograms were run with tert-amyl alcohol saturated with buffer (17). *p*-Aminobenzoic acid and cyanogen bromide were the spot reagents. In some cases, the chromatograms were cut into strips and scanned for radioactive spots with a windowless, flowing-gas proportional counter attached to an automatic strip chart recorder.

PREPARATION AND ADDITION OF NICOTINES: Pyrrolidine-C^{14}-nicotine was prepared by supplying 9.2 mg of DL-ornithine-2-C^{14} monohydrochloride (2.84×10^7 dpm/mg C) as obtained from Tracerlab to 25 cultures of the excised roots of *Nicotiana tabacum* var. Turkish. After 18 days, the nicotine was isolated from these cultures as described above. This nicotine had an activity of 1.36×10^6 dpm/mg C. The radiochemical yield was 0.11. Dewey, Byerrum, and Ball (10) and Leete (13) have shown that the activity contributed by ornithine-2-C^{14} to nicotine is located almost entirely in the pyrrolidine ring.

Pyridine-C^{14}-nicotine was obtained by supplying to similar root cultures of *N. tabacum* nicotinic acid ring-labeled with C^{14} by nuclear recoil methods (1, 9, 21). A total of 3.75 mg (sp. act. 1.93×10^8 dpm/mg C) was added to 25 cultures. The nicotine produced in the presence of this acid possessed an activity of 1.86×10^2 dpm/mg C. It has been shown elsewhere (9) that pyridine ring label of nicotinic acid is recoverable as pyridine ring label of nicotine.

Pyrrolidine-C^{14}-nicotine (10.1 mg and 5.07×10^4 dpm) was supplied as the hydrochloride to 79 cultures of excised roots of *N. glauca* for 38 days. Likewise, pyridine-C^{14}-nicotine (8.6 mg and 6.85×10^2 dpm) was supplied, also as the hydrochloride, to another 79 cultures for 38 days. Each group of cultures was harvested in two approximately equal sub-

lots. The alkaloid picrates were isolated and assayed for C^{14} as described above.

Nonlabeled nicotine was prepared by steam distilling nicotine dipicrate (Distillation Products Indus.) into HCl. The alkaloid was supplied as the hydrochloride (pH 4.0) to two lots of 50 cultures each. Two lots of cultures, 50 each, served as controls.

GROWTH MEASUREMENTS: Growth of the roots in sterile culture was measured by weighing the material obtained at each harvest after drying in a ventilated oven at 60° C.

RESULTS

ALKALOID PRODUCTION RATES: In the early part of the culture period, the roots made anabasine and nicotine in the proportion of 2 to 1 (fig 1). Toward the end of each passage, however, the ratio had changed to 4 to 1. These figures fall in the range already established for relative nicotine and anabasine production capacities of *N. glauca* roots as estimated by the use of reciprocal grafts (6).

Actual production of nicotine increased exponentially with elapsed time during the first 20 days as did also root dry weight. For reasons outlined by Solt (16), however, it is convenient to eliminate the time variable and to consider directly the linear relation that exists between nicotine production and root dry weight. In the present instance, once the cultures in two different passages began to grow at an exponential rate, namely, at 10 to 15 days from the initial inoculation up to the 40th day, the yield of nicotine was 2.72 ± 0.214 μg per mg root dry weight (statistic is standard error of estimate). This figure is approximately 1/11th the constant relating nicotine yield to growth in root cultures of *N. tabacum.*

Solt has shown that the individual branch of cultured roots of *N. tabacum* grows and produces nicotine at constant rates (16). Since growth occurs at or near the tip of the individual root branch, it seems reasonable to conclude that nicotine production may also occur there and that the rate dependency of nicotine production upon growth may rest upon restriction of both processes to a common morphological compartment. Solt has, in fact, shown experimentally that this is the case in the root tip of *N. tabacum,* and there seems to be no doubt that the same conclusion may be drawn in the case of *N. glauca.* Conceptually, the Nicotiana root tip may thus be regarded as a moving point source for both new tissues and nicotine.

Actual production of anabasine did not follow a simple relationship with growth in terms of dry weight

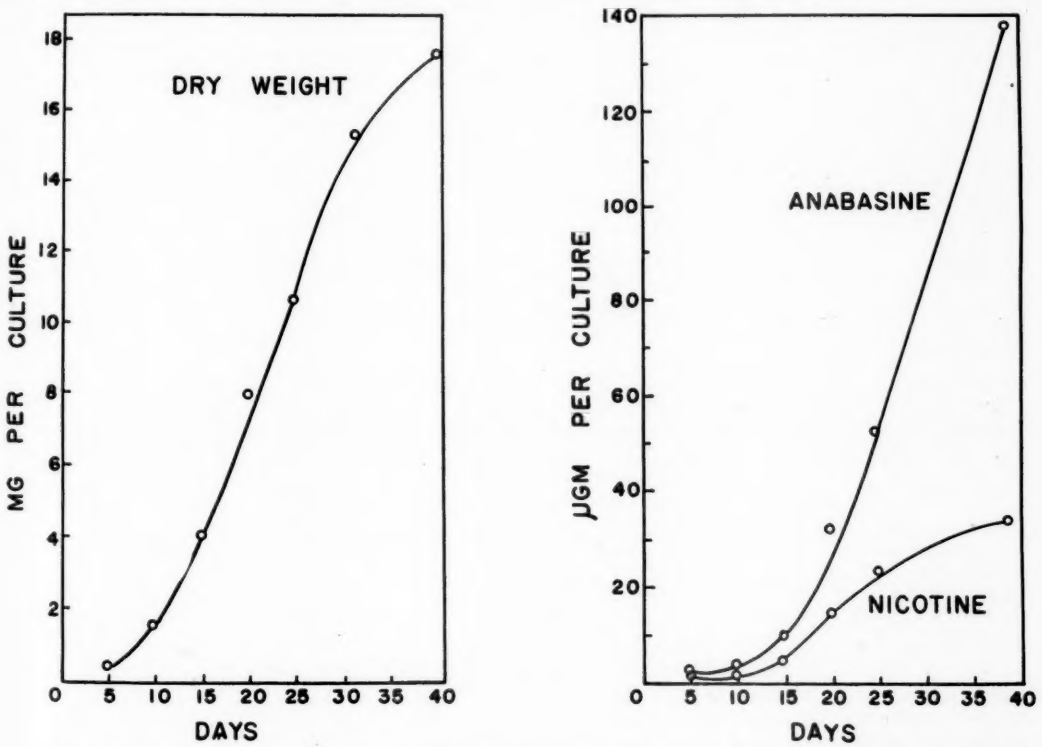

FIG. 1. Mean dry weight and alkaloid yields of excised root cultures of *Nicotiana glauca* in the course of one passage.

increase. Instead, the production of this alkaloid occurred at a progressively faster rate than that of root dry weight and continued at a relatively high level even after growth rate had become virtually nil in the last stages of each passage. The implication that anabasine production may be a property of matured root tissues was examined in the following way. It was assumed A: that the compartment for anabasine production is the entire root organ possibly but not necessarily exclusive of the growing tips; B: that production of the alkaloid proceeds at a uniform rate throughout all parts of the compartment and for the duration of the culture period, and C: that growth proceeds at a relatively constant rate also during this period. The amount of anabasine produced in any given period of time would, therefore, be proportional both to the amount of elapsed time and to the total mass of matured root tissues. Obviously, if the third assumption is even approximately true, the specific relationship between these three variables may be stated as follows. At any given moment the anabasine content of a root culture in micrograms is equal to a proportionality constant multiplied by the product of the root dry weight in milligrams and the half-time of the culture in days. When this calculation is made for the period 10 to 40 days, there is obtained a proportionality constant of 0.40 ± 0.008. The very small standard error of estimate indicates a small amount of dispersion about the calculated line of regression and therefore validates the suggested relationship. It is necessary only to comment that growth follows a sigmoid relationship with respect to time and is not strictly linear as assumed. The shape of the curve is such, however, that departure from linearity is not great. Thus, the line of least squares relating these two variables possesses a slope of 0.584 mg per day with a standard error of estimate ± 0.037. The latter figure is equivalent to a coefficient of variation 6.34 %. The fact that nicotine and anabasine synthesis may occur in different compartments is of interest in connection with the use of different precursors for these syntheses as described below.

INCORPORATING LABELED PRECURSORS: In an earlier paper (9) we have described the incorporation of labeled nicotinic acids into the pyridine ring of nicotine by excised root cultures of *N. tabacum*.

In the present case, 2-tritium-nicotinic acid was incorporated into both nicotine and anabasine, resulting in radiochemical yields (table I) commensurate with the relative levels of production of these two alkaloids. Oxidation of one preparation of tritium-labeled anabasine to nicotinic acid gave a recovery of 88 % of the tritium contained in the anabasine. It is clear that most of the nicotinic acid ring label had entered the pyridine ring of anabasine. Evidently, the pyridine rings of nicotine and anabasine both originate with nicotinic acid in *N. glauca*.

The pathway of conversion of nicotinic acid pyridine ring to nicotine pyridine ring was shown earlier (9) to involve loss not only of the carboxyl group but also of the hydrogen atom in the 6 ring position. When nicotinic acid labeled with tritium in position 6 was supplied to root cultures of *N. glauca*, only 1.1 % of the radioactivity was recovered as labeled nicotine and anabasine (table I).

Ornithine has been shown to provide the pyrrolidine ring of nicotine in *N. tabacum* (10, 13). Ornithine-2-C^{14} supplied to the glauca cultures gave more than 15 times as much label in the nicotine subsequently produced as in the anabasine (table I). Despite this expected outcome, actual radiochemical yields were much lower than expected. Possibly in *N. glauca* ornithine is more rapidly metabolized by other pathways than appears to be the case in *N. tabacum*. Leete (14) has shown that lysine is the precursor of the piperidine ring of anabasine. Tests conducted in our sterile root culture system confirmed this result, for 100 times as much label was introduced into anabasine from lysine than into nicotine (table I). It may be concluded, therefore, that nicotine biosynthesis in *N. glauca* very likely follows the same pathway as it does in *N. tabacum*. The anabasine biosynthesis pathway shares with the nicotine path-

TABLE I

INCORPORATION OF LABELED INTERMEDIATES INTO ALKALOIDS BY EXCISED ROOTS OF NICOTIANA GLAUCA

COMPOUND SUPPLIED			PERIOD OF CONTACT	ALKALOID YIELDS*		RADIOCHEMICAL YIELDS	
COMPOUND	SPECIFIC ACTIVITY	CONCENTRATION**		ANABASINE	NICOTINE	ANABASINE	NICOTINE
	dpm/mg	mg/ml	DAYS	mg	mg		
			Subclone A				
Nicotinic acid-H^3	265,000	0.005	21	14	7	0.22	0.05
Nicotinic acid-2-H^3	257,000	0.007	39	51	15	0.42	0.12
Lysine-C^{14}	333,000	0.0017	26	10	7	0.29	0.003
			Subclone B				
Nicotinic acid-2-H^3	221,000	0.012	42	78	23	0.08	0.02
Nicotinic acid-2-H^3	221,000	0.020	45	37	17	0.05	0.02
Nicotinic acid-6-H^3	172,960	0.0097	34	108	35	0.008	0.003
DL-Ornithine-2-C^{14}	8,300,000	0.009	27	51	24	0.001	0.015

* As the crystalline dipicrates.
** In the culture medium (total vol/culture, 30 ml).

way a similar use of nicotinic acid; but, as Leete has shown, it departs from the latter in using a homolog of ornithine, namely lysine, as the precursor of the piperidine ring.

One interesting point may be noted in passing. Subclone A gave extraordinarily high radiochemical yields and specific activities when supplied with labeled alkaloid precursors (table I). This unusual correlation between morphological and biochemical parameters merits further consideration in terms of the identification of the factors which are rate-limiting for alkaloid synthesis in these excised root systems. In the latter connection, note that none of the alkaloid precursors supported an increase in nicotine or anabasine yield. Apparently, the labeled intermediates were incorporated into nicotine and anabasine simply by pool dilution.

Nicotinic Acid Content of Root Tissues: Total acid-hydrolyzable nicotinic acid content of the root cultures (table II) increased exponentially with elapsed time during the first 20 days of each passage when the cultures were transferred at sufficiently short intervals (21 days) to maintain both growth and alkaloid production under steady state conditions (cf. 16). The proportionality constants (logs) for growth in dry weight (0.078) and for nicotinic acid content (0.072) were essentially equal. Therefore, it is convenient once more to consider chemical composition as a simple linear function of root dry weight. The proportionality constant relating mean nicotinic acid content per culture to mean root dry weight per culture was 0.127 ± 0.009 μg per mg.

Since nicotinic acid is a precursor of both anabasine and nicotine, it seems reasonable to combine the yields of both into a single figure for alkaloid yield by the root culture of *N. glauca*. At 40 days of age, the culture had produced alkaloids (anabasine plus nicotine) at an average rate of 9.78 μg per mg root dry weight. The comparable figure for nicotine yield by the excised root culture of *N. tabacum* is 29.05 μg per mg. The ratio between these two figures is 2.95. The nicotinic acid content of the *N. glauca* root culture was 0.127 μg per mg root dry weight. Again, the comparable figure for tabacum is 0.298 μg per mg. The ratio between these two figures is 2.35. Obviously, the concentration of total available nicotinic acid (by acid hydrolysis) in the tissues of these two types of roots is rather closely related to the actual amounts of pyridine alkaloids which they produce. Whether or not this correlation would extend to other comparisons within the genus Nicotiana is unknown.

When nicotinic acid (337 μg/culture) was supplied to *N. glauca* root cultures on the 8th day of the passage, 210 μg was recovered in the culture medium on the 20th day. Nine μg were recovered from the roots. At 20 days, one single root culture not supplied with nicotinic acid would have contained about one μg of nicotinic acid (cf. above). Consequently, a net recovery of 218 μg was realized. The difference, 120 μg per culture or 36 % of the original amount supplied, was presumably metabolized by the roots. Radiochemical yields calculated on the basis of amounts of labeled nicotinic acid which disappeared will obviously be somewhat greater than those listed in table I.

Although labeled nicotinic acids supplied to the root cultures were always incorporated into nicotine, the failure of such additions to affect nicotine or anabasine yields in the quantitative sense is noteworthy as has been indicated above. The present observations indicate quite clearly that, whether nicotinic acid is supplied to the cultures or not, there does not occur a rate-limiting step for alkaloid production anywhere in the reaction sequence up to and including nicotinic acid formation. In this sense, the observed correlation between total alkaloid production and nicotinic acid content of the root tissues of the species under discussion (see above) is entirely coincidental.

Attempted Alteration of Relative Rates of Anabasine and Nicotine Production: Lysine and ornithine were supplied separately to the root cultures with and without nicotinic acid, and the ratios of anabasine yield to nicotine yield were calculated (table III).

The customary statistical analysis of these ratios revealed the occurrence of wholly negligible variance for the comparison nicotinic and no nicotinic acid and for interaction between pyridine and pyrrolidine-piperidine precursors. Variance for the comparison pyrrolidine-piperidine with no pyrrolidine-piperidine precursors yielded a ratio of 0.81 in the F-test with the variance within treatments. Even this falls far short of the value 19.37 required for significance at

Table II

Nicotinic Acid Content of Root Cultures With and Without Added Nicotinic Acid

Treatment	Days	Mean root dry wt	Mean nicotinic acid content/culture	
			Roots	Culture medium
		mg	μg	μg
None	8	1.10	0.150	1
	11	2.30	0.250	...
	14	4.70	0.575	...
	17	7.83	0.90	...
	20	8.87	1.18	...
Nicotinic acid added (337 μg/culture)	20	9.4	9.15	210

TABLE III

ALKALOID CONTENT OF ROOT CULTURES SUPPLIED WITH NONLABELED PRECURSORS

TREATMENT	MEAN DRY WT OF ROOTS	MEAN CONTENT/CULTURE		CONC IN ROOTS		RATIO OF ANABASINE TO NICOTINE
		NICOTINE	ANABASINE	NICOTINE	ANABASINE	
	mg	μg	μg	μg/mg	μg/mg	
No additions	19.6	43	105	2.2	5.4	2.4
	19.0	42	121	2.2	6.4	2.9
Ornithine	23.6	53	165	2.2	7.0	3.1
Lysine	23.3	58	181	2.5	7.8	3.1
	22.4	60	160	2.7	7.1	2.7
No additions	26.3	83	189	3.2	7.2	2.2
	24.8	63	166	2.5	6.7	2.7
Ornithine + nicotinic acid	26.2	77	198	2.9	7.6	2.6
	25.7	57	184	2.2	7.2	3.2
Lysine + nicotinic acid	25.7	80	234	3.1	9.1	2.9
	28.7	78	257	2.7	9.0	3.3
Nicotinic acid	...	71	241	...	...	3.4
	...	56	126	...	...	2.3
	...	91	281	...	...	3.1

the 95 % level of probability and the prevailing degrees of freedom (8 and 2).

It might have been supposed, since nicotinic acid (the common precursor) concentration is evidently not a limiting factor, that the production of one or the other alkaloid could have been favored by supplying its specific precursor in excess. Such expectation was not realized. Possibly, lack of interplay between the two alkaloid-producing mechanisms may have resulted from their separation into different compartments within the root (cf. above). Possibly, also, there are specificities in the pathways of synthesis of the two alkaloids which preclude such interplay. Leete (14) has shown, for example, that lysine is converted to anabasine via an unsymmetrical intermediate, whereas he and Lamberts et al (12, 15) have shown that ornithine traverses a symmetrical intermediate on the pathway to nicotine. Whatever the reason, the stability of alkaloid production rates in this experimental system is extraordinary and deserving of further experimental attention.

METABOLISM OF ADDED NICOTINES: Pyridine-C^{14}-nicotine was recovered from the root cultures to the extent of 60 % of the amount originally added. The corresponding figure for pyrrolidine-C^{14}-nicotine was only 43 % (table IV). The chemical basis for a differential turnover rate for the two rings is not known but may hold considerable interest for future investigation.

The above figures include some radioactivity that appeared in the anabasine fraction from the SuperCel columns. However, examination of paper chromatograms made from the alkaloid fractions as they came off the columns indicated the presence of radioactivity not in anabasine but in the small amounts of nornicotine that accompanied anabasine. Experiments in which nonlabeled nicotine was added to similar cultures revealed that the quantities of nornicotine did not increase as a consequence of nicotine feeding (table V). Therefore, the appearance of activity in nornicotine indicates ready transfer of methyl groups between pyridyl-pyrrolidyl residues without concomitant changes in the actual pool size of nornicotine. For this reason, and because of the known biosynthetic

TABLE IV

RECOVERIES OF RADIOACTIVITY AFTER SUPPLYING NICOTINE LABELED WITH CARBON 14 IN PYRIDINE AND PYRROLIDINE RINGS, RESPECTIVELY, TO EXCISED ROOT CULTURES OF NICOTIANA GLAUCA

COMPOUND SUPPLIED	AMOUNT SUPPLIED	ALKALOID YIELDS**		RADIOCHEMICAL YIELDS*
		ANABASINE	NICOTINE	
	mg	mg	mg	
Nicotine-pyridine-C^{14}	3.96	2.8	3.4	0.69
" " "	3.47	2.9	2.7	0.51
Nicotine-pyrrolidine-C^{14}	4.14	3.4	3.4	0.43
" " "	3.92	4.6	3.8	0.44

* Includes all radioactivity recovered in nicotine and nornicotine (cf. text).
** As free bases.

relationship between nicotine and nornicotine (7), the recoveries of radioactivity listed above may, for the present purpose, be assigned to nicotine.

When nonlabeled nicotine was added to the cultures (table V), 74 % was recovered after 38 days. This quantity is higher than the recovery of pyridine-ring-labeled nicotine given above. However, the concentration of added alkaloid in the tracer experiment was half that employed in the present experiment. The mean amounts of nicotine to disappear per culture were 55 μg in the latter case and 42 μg in the former. Evidently, the fraction of added nicotine to disappear is related to the amount originally supplied. In the experiments described here, the amounts of nicotine added to the culture solutions were of the order of magnitude of the amounts normally contained in the cultures at the end of a passage. Consequently, the losses described herein may be expected to exceed those which would occur in the root culture to which no nicotine is added.

Discussion

The unity of alkaloid biosynthesis in the Nicotiana genus is indicated in the present instance by the utilization of nicotinic acid as precursor of the pyridine rings of nicotine and of anabasine. Variations in the reduced ring were accounted for by using ornithine and its homolog lysine as precursors. The latter results confirm in a microbially sterile system the results already reported in greater detail by Leete (13) and by Dewey, Byerrum, and Ball (10) using whole tobacco plants.

By employing a system in which both nicotine and anabasine are produced concurrently, it has been possible to show that the two alkaloids are probably synthesized in separate portions of the root mass. Nicotine production, strictly correlated ratewise with growth, occurs in the root tips, while anabasine production seems to occur primarily in matured root tissues. Nicotine production in the excised root of *N. tabacum* also occurs in the root tip where growth is a prominent physiological activity. The rate of production of nicotine in both systems is so closely dependent upon growth rate as to suggest an obligatory dependency. In chemical terms, this would mean that the rate of a single chemical reaction is limiting for both processes. Consequently, nicotine production becomes a facet of the biochemistry of growth, a rather new notion in the field of plant physiology. It is tempting to speculate that successful identification of the rate-limiting step for nicotine production might open a backdoor into the still obscure biochemistry of plant growth and development.

Evidently, the rate-limiting step for nicotine production must lie at a point on the sequential reaction pathway between nicotinic acid plus ornithine or lysine and nicotine or anabasine, respectively. There is no present indication of the identity of this rate-limiting step, although several obvious possibilities suggest themselves for experimental test. One is the production rate of a postulated 1,6-dihydronicotinic acid (8) and the other is the production rate of the piperidine or pyrrolidine ring precursor from lysine or ornithine, respectively.

The rate stability of anabasine production parallels that of nicotine production (8, 16). Failure to obtain a diversion of alkaloid synthesis from one to the other by adding lysine and ornithine separately is a case in point. It may be that the same step is rate-limiting for both alkaloids. If so, it is difficult to understand why nicotine production should be so intimately related to growth rate while anabasine production is not. By way of speculation, the unusual regenerative properties of *N. glauca* may be cited (11). It seems entirely possible that matured root tissues of this species could retain a limited portion of the sequential reaction pathways necessary for growth or development sufficient to permit continued pyridine alkaloid production.

There is an appreciable turnover of nicotine in these root cultures, but the exact magnitude cannot be estimated from the data given. Owing to the exponential nature of growth and of nicotine accumulation, it is evident that nicotine turnover would best be measured by adding small amounts of nicotine of high specific activity at intervals throughout the culture period. Such intervals and the amounts of material to be added should be so chosen as to coincide approximately with the course of nicotine accumulation in the culture.

In conclusion, the excised root cultures of *Nicotiana glauca* afford an excellent experimental system for investigating the kinetics, the compartmentaliza-

Table V

Effects of Added Nicotine Upon Alkaloid Composition of Excised Root Cultures of Nicotiana Glauca

Treatment	Mean dry wt of roots/culture	Alkaloid content/culture		
		Nicotine	Anabasine	Nornicotine
	mg	μg	μg	μg
No additions	22.6	68	164	4
" "	21.5	67	165	5
Nicotine added (213 μg/culture)	23.2	200	172	4
Nicotine added (213 μg/culture)	23.1	207	154	6

tion and the pathways of pyridine alkaloid biosynthesis. The kinetics and the sites of synthesis are quite distinct in the two cases, anabasine and nicotine. So, also, are the pathways of their biosynthesis beyond the step in which nicotinic acid is prepared for reaction with the piperidine or the pyrrolidine ring precursor. The nature of the link between the process by which these alkaloids originate and the fundamental growth chemistry of the plant offers a new and stimulating subject for further study.

Summary

The simultaneous production rates of anabasine and of nicotine by excised root cultures of *Nicotiana glauca* Grah. have been analyzed in relation to root growth rate. It is shown that nicotine production is simply proportional to root dry weight yields but that anabasine production is proportional to the product of root dry weight and the length of the culture period. These relationships are interpreted to indicate a restriction of anabasine and nicotine synthesizing functions to different compartments in the root system.

By using isotopically labeled nicotinic acids it has been shown that the pyridine ring of anabasine and very likely also that of nicotine are produced in the manner already demonstrated for the origin of nicotine in *N. tabacum*. This transformation involves loss of the hydrogen atom in position 6 of the pyridine ring of nicotinic acid.

The preferential incorporation of labeled ornithine into nicotine and of labeled lysine into anabasine was interpreted to afford confirmation, in a microbially sterile system, of the prior observations of R. U. Byerrum and of E. Leete and their coworkers.

The tissue concentrations of nicotinic acid during growth of the root cultures was followed by a microbiological method of assay. Nicotinic acid content of the roots was simply proportional to root dry weight. Of the nicotinic acid added to the cultures, 64 % was recovered by microbiological assay. It was concluded, therefore, that the concentrations of nicotinic acid as employed in our tracer experiments have not been rate-limiting for alkaloid biosynthesis.

It was not possible to increase the chemical yields of anabasine or of nicotine or to influence the relative proportions in which they are produced by supplying nicotinic acid alone or in combination with ornithine or lysine. The rate stability of alkaloid biosynthesis in this experimental system was emphasized.

By adding labeled nicotine to the cultures, it was shown that there is some turnover of this alkaloid. The losses lay between 26 and 40 % at the dosage levels employed.

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ACCUMULATION AND TRANSFORMATION OF SUGARS IN SUGAR CANE STALKS[1]

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Previous studies by Hartt (4) and Bieleski (1) on the accumulation and transformation of sugars in sugar cane stalks have demonstrated the interconversion of glucose and fructose, and the formation of sucrose from reducing sugars. An enzyme prepared from sugar cane shoots or roots (2) was shown to catalyse a reaction between uridine diphosphoglucose and fructose, yielding sucrose. The enzyme sucrose phosphorylase has been reported in sugar cane leaves (6).

Sugar is taken into stalk tissue slices by two processes (1). The first process reaching equilibrium within 30 minutes was proportional to the external sugar concentration, was unaffected by anaerobic conditions, and was completely reversed when the tissue was placed in water. The sugar diffuses to and from a portion of the tissue, subsequently termed the "outer space," and the "external solution". The second process continued at a constant rate for up to 60 hours; was inhibited by anaerobic conditions, and was independent of the concentration at levels above 0.2 %. This process accumulated sugar against a large concentration gradient and is subsequently referred to as "accumulation into the inner space". The kinetics of accumulation were similar for glucose, fructose, and sucrose, and corresponded to those for enzymes described in the Michaelis-Menten system. The sugar accumulated into the inner space was mainly sucrose regardless of which of the sugars was supplied in the external medium.

A number of studies have been made on the uptake and transformation of sugars in other higher plants (3, 5, 7). Edelman, Ginsberg, and Hassid (3) found that radioactive glucose and fructose were rapidly absorbed by wheat seedlings giving labelled hexose phosphates, sucrose, and cellulose. When glucose-1-C^{14} was supplied, the glucose and fructose moieties of accumulated sucrose were approximately equally labelled, and most of the activity resided in the C-1 of both the hexose residues. Similarly, the activity in hexose phosphates and cellulose was mainly in the C-1 position, indicating that hexoses are directly incorporated into complex saccharides. Working with Canna leaf discs, Putman and Hassid (5, 7) concluded that sucrose uptake is preceded by hydrolysis at the cell surface.

The work reported here extends certain aspects of the previous work on sugar uptake and transformation in sugar cane stalks.

MATERIALS AND METHODS

The sugar cane variety H33/1937 was used throughout these investigations. The cane was approximately one year old and, for experiments with excised tissue, was cut and used immediately. The rind was first removed and tissue discs 5.0 mm in diameter and 0.5 mm thick, cut transversely with a sharp cork borer and a hand microtome. The discs were washed in running water for 60 to 90 minutes prior to immersion in sugar solutions, contained in 30 ml conical flasks, the flasks being capped with aluminum foil and placed in a shaker at 60 oscillations per minute and at 25° C. Increasing the shaking speed to 120 oscillations per minute did not increase the rate of sugar uptake. After immersion, the sugars in the outer space were removed by washing the discs in running water for 60 minutes (see table I).

Radioactive sucrose and glucose were obtained from Isotopes Specialties Co., California. Sugars were extracted from the tissues with 3 volumes 96 % ethanol, and separated using descending chromatography on Whatman No. 1 paper and ethyl acetate, pyridine and water (8:2:1), or *n*-propanol, water and ethyle acetate (7:2:1) as developing solvents. Marker strips were always run. In some experiments, assays of radioactive sugars were made direct-

TABLE I

REMOVAL OF OUTER SPACE SUGARS FROM TISSUE DISCS

WASHING TIME MIN	% OF OUTER SPACE SUGAR REMAINING IN DISCS
Drained and blotted	100
1	9.8
4	3.4
11	1.8
15	1.4
25	0.5
60	−0.1
90	0.0

Replicate batches of 25 tissue discs were incubated in 1.0 ml C^{14}-labelled sucrose (1.0 mM/ml) for 90 minutes. The discs were removed and washed with running tap water for the periods stated, then extracted with 3 volumes 96 % ethanol and the C^{14} content of the extract determined.

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ly on the paper, after detection with *p*-anisidine phosphate. The radioactivity assay was unaffected by the color reagent. In other experiments, where activities were low, the sugars were eluted and counted on planchets. All assays for radioactivity were made with a thin end-window Geiger-Muller tube.

Results

Comparison of Labelling Patterns of Inner Space Sugars Derived Either From Photosynthate or From Sucrose Supplied in Solution: $C^{14}O_2$ was fed to the seventh leaf (counting down from the spindle) of an intact plant, and photosynthesis and translocation allowed to proceed for a 24-hour period. Tissue slices were then cut from the fifth, seventh, and tenth internodes, washed and divided into two lots for each internode. One lot was extracted immediately with 3 volumes 96% ethanol. The second lot was placed in C^{14}-labelled sucrose for 4 hours. The outer space sugars were removed and the tissues extracted for analysis. The distribution of C^{14} among the sugars (cf. table II) shows that accumulation into the inner space gives similar results whether the source of sugar is from the translocation of photosynthate or from sucrose contained in a solution bathing tissue discs.

Comparison of Rates of Active Accumulation of Sucrose and Glucose Into Inner Space of Tissue Discs From Internodes of Different Ages: The young internodes of commercially-grown varieties of sugar cane contain relatively large amounts of the reducing sugars, glucose and fructose, while the mature internodes contain scarcely any. Climate and physiological age of the plant affect the general picture.

Bieleski (1) measured the disappearance of sugar from a solution bathing sugar cane tissues (i.e., the net rate of uptake), and observed that mature internodes had much lower rates of accumulation than young internodes, in fact tissue from mature internodes of old stalks showed no sugar uptake over a 70-hour period. The net rate of uptake was found to decline as the sugar concentration of the tissue increased.

Table III

Accumulation Rates of C^{14}-Labelled Glucose and Sucrose Into Inner Space of Internodal Tissue Discs

Internode	Sucrose uptake μM g dry wt/day	Glucose uptake μM g dry wt/day	Sugar content of tissue: Sucrose % dry wt	Sugar content of tissue: Reducing Sugars % dry wt
5	3.65	30.8	11.5	15.7
7	2.76	26.8	34.6	7.8
10	0.61	4.9	55.2	1.6
15	0.61	2.1	54.5	0.36

25 tissue discs were placed in 1.0 ml. solution containing either 1.0 μM C^{14}-labelled sucrose or glucose and incubated for 4 hours at 25° C. The outer space sugars were removed and the inner space sugars extracted in 3 volumes 96% ethanol for assay of radioactivity. Sugar contents were determined on separate samples.

Further investigations of uptake in young and mature tissues have been made by following the accumulation of isotopically labelled sugars into the inner space. Consider the reaction sequence:

outer space sugar —(active accumulation)→ inner space sugar ⟷(diffusion) outer space sugar

In a short-term experiment, where the amount of labelled sugar entering is small compared with the amount of unlabelled sugar in the inner space, the effect of any back diffusion may be neglected. The rate of uptake of isotope will then be a close approximation of the initial rate of active accumulation. Glucose and sucrose uptake measured in this way (table III) declined, though disproportionately, from the

Table II

Distribution of C^{14} in Sugars in Internodal Tissues When Isotope is Derived From Translocation of Photosynthate or From Sucrose Contained in Solution Bathing Tissue Discs

Internode	Source of C^{14}	Activity in extract DPM	% Distribution of activity: Sucrose	Glucose	Fructose	Sucrose activity as % of activity in sugars
5	PS*	1,680	51	7	9	76
5	PS + sucrose solution	20,680	61	3.7	1.6	93
7	PS	16,400	65	1.2	1.5	96
7	PS + sucrose solution	28,000	70	2.0	1.4	95
10	PS	14,700	64	1.5	...	96
10	PS + sucrose solution	20,000	76	3.0	3.0	84

* Photosynthesis.

$C^{14}O_2$ was fed to the seventh leaf, and photosynthesis and translocation allowed to continue for 24 hours. Tissue discs were cut, washed, and divided into two lots. One lot was extracted immediately; the second was placed in 1.0 ml C^{14}-labelled sucrose (1.0 μM/ml) for 4 hours, the outer space sugar removed, and the tissue extracted. The sugars were separated chromatographically prior to assay.

TABLE IV

C¹⁴ DISTRIBUTION IN SUGARS FROM INNER SPACE OF INTERNODAL TISSUE DISCS INCUBATED IN C¹⁴-LABELLED GLUCOSE OR SUCROSE

INTERNODE	SUGAR SUPPLIED	DISTRIBUTION OF C¹⁴ AS % OF TOTAL ACTIVITY IN EXTRACT		
		SUCROSE	GLUCOSE	FRUCTOSE
5	Glucose	77	1	2
5	Sucrose	79	2	...
7	Glucose	66	2	...
7	Sucrose	75	2	...
15	Glucose	47	16	1
15	Sucrose	76	...	...

Experimental detail described under table III.

5th to the 15th internode. Therefore, the fall in the net rate of sugar uptake as the tissues mature is partly due to a decline in the rate of influx, and not solely due to a higher efflux because of the increased concentration gradient.

Analyses of the distribution of C^{14} among the sugars of the inner space (cf. table IV) show that, for young internodes, sucrose was accumulated whether or not glucose or sucrose was supplied in the bathing solution. The sucrose synthesized from labelled glucose in the seventh internode contained approximately equal amounts of label in the glucose and fructose moieties (table V). In young internodes there is a large amount of fructose, and the lack of utilization of this sugar in sucrose synthesis indicates that conversion of glucose to sucrose occurs at a site separated from the inner space. A similar observation has been recorded for Canna leaf discs (3).

In older tissue from internode 15, sucrose was accumulated as such, but when glucose was supplied 25 % of the label in the inner space sugar was in glucose and 74 % in sucrose. Also the sucrose synthesized from glucose was predominantly labelled in the glucose moiety (tables IV and V). These results show a lowered capacity in older tissues to synthesize sucrose from glucose, possibly because of inability to convert glucose to the fructose moiety at a sufficient rate.

MOVEMENT OF SUGARS OUT FROM INNER SPACE OF INTERNODE TISSUE DISCS: C^{14} was introduced into the sugars of the inner space by supplying $C^{14}O_2$ to the seventh leaf of a growing plant and allowing photosynthesis and translocation to proceed for a 24-hour period. Analyses were then made of the sugars in the internodes and, at the same time, tissue discs were placed in water and analyses made of the sugars diffusing into the water. The distribution of

TABLE V

C¹⁴ DISTRIBUTION IN HYDROLYSIS PRODUCTS OF SUCROSE FROM INNER SPACE OF INTERNODAL TISSUE DISCS INCUBATED IN C¹⁴-LABELLED GLUCOSE

INTERNODE	C¹⁴ IN HYDROLYSIS PRODUCTS OF SUCROSE AS % OF TOTAL ACTIVITY	
	GLUCOSE	FRUCTOSE
7	54	46
15	73	27

System as in table III. The sucrose was separated by paper chromatography, eluted, hydrolysed, and the hydrolysis products separated chromatographically prior to assay.

C^{14} in the diffusate (cf. col. 7, 8, and 9, table VI) may be quite different from that in the inner space (col. 4, 5, and 6). For immature tissues the diffusate is diminished in sucrose and enriched in fructose, while in mature tissues it is enriched in fructose. If it is assumed that the C^{14} in the sugars in the diffusate

TABLE VI

C¹⁴ DISTRIBUTION IN INNER SPACE OR MOVING FROM INNER SPACE OF INTERNODAL TISSUE DISCS FROM PLANT FED $C^{14}O_2$

INTERNODE	SUGAR CONTENT OF JUICE mg/ml		DISTRIBUTION OF C¹⁴ IN SUGARS IN JUICE % OF TOTAL			DISTRIBUTION OF C¹⁴ IN SUGARS MOVING FROM DISCS % OF TOTAL		
	SUCROSE	REDUCING SUGARS	SUCROSE	GLUCOSE	FRUCTOSE	SUCROSE	GLUCOSE	FRUCTOSE
5 & 6	4.5 (450)*	14 (49)	63	13	10	20	8	48
7 & 8	16 (525)	19 (20)	92	2	2	47	3	19
9 & 10	49 (140)	17 (9)	87	...	2	86	...	7

* Figures in parentheses are activities as dpm/mg sugar.

The seventh leaf of a growing plant was fed 1.0 millicurie of $C^{14}O_2$, and photosynthesis and translocation allowed to proceed for 24 hours. Juice samples were pressed from internodal tissues at a pressure of 1,000 lb/sq inch. Approx. 100 mg dry weight tissue discs were washed in running water for 1.5 hours, then placed in 5.0 ml water and shaken gently for 4 hours at 25° C. The ambient solution was removed for analysis.

reflects the permeability of the barrier separating the inner and outer space, the relative permeability of the barrier to sucrose and reducing sugars may be estimated. For the conditions of the experiment, the sugar concentration in the diffusate may be neglected as being very small compared with that in the tissue. Using Fick's Law, the ratio of the diffusion constants for reducing sugars and sucrose is given by $\frac{D_1}{D_2} = \frac{M_1 C_2}{M_2 C_1}$ where D_1 and D_2, C_1 and C_2, M_1 and M_2 are the diffusion constants, the tissue concentrations and the amounts of reducing sugar or sucrose, respectively, which move to the external solution over the time course of the experiment.

The values of D_1/D_2, calculated from table VI, are eight for internodes 5 and 6, ten for 7 and 8, and four for 9 and 10, and indicate that the barrier is considerably more permeable to reducing sugar than to sucrose, depending on the age of the tissue. Also the analytical data suggest that permeability to fructose is higher than to glucose.

The rates of efflux of total sugars from tissue slices are given in table VII, and were calculated from the C^{14} assays of the diffusate and the specific activity data obtained from analyses of sugars in the tissues. A minimum rate of efflux occurs in the internodes which are just ceasing rapid elongation. The fall in the efflux rate from internode 5 to 7, which occurs while the concentration of reducing sugar rises to a maximum and the concentration of sucrose is also rising, indicates that the permeability of the tissues is reduced as they mature. The subsequent rise in the rate of efflux is consistent with an increasing concentration of sugar in the tissue, but the observed rates are considerably greater than would be expected on the basis of the gradient alone.

Thus, maturation of the tissue is accompanied by both an increase in the rate of efflux and a reduction in the rate of accumulation.

Table VII

Rates of Efflux of Sugar From Internodal Tissue Discs

Internode	Sugar content % fresh wt	Efflux rate mg sugar/g fresh wt/day
5	1.55	2.3
6	2.02	1.1
7	2.61	0.41
8	3.74	1.6
9	4.95	5.6
10	8.25	11.0

Experimental detail described under table VI.

Table VIII

Unlabelled Sucrose Effect in External Medium on Rate of Efflux of C^{14}-Labelled Compounds From Internodal Tissue Discs

Composition of external medium	C^{14} Efflux to medium dpm/5.0 ml
Water	1,680
0.02 % sucrose	1,700
0.20 % sucrose	1,780
2.00 % sucrose	1,570

1.0 millicurie $C^{14}O_2$ was fed to the sixth leaf of a growing plant and photosynthesis and translocation allowed to proceed for 24 hours. Tissue discs were cut, washed in running water for 1.0 hour, then placed in 5.0 ml water or sucrose solution. After 4 hours incubation at 25° C, the tissue was removed and aliquots of the medium dried on planchets for counting. The sucrose concentration was adjusted to give similar self-adsorption characteristics in each treatment.

Effect of Unlabelled Sucrose in External Medium on Rate of Efflux of C^{14}-Labelled Compounds From Internodal Tissue Slices: The net rate of change of sugar concentration in the inner space should be the algebraic sum of the accumulation and efflux rates. However, a mechanism can be conceived in which the presence of sugar in the outer space would prevent the efflux of sugar from the inner space.

To test this possibility, $C^{14}O_2$ was fed to an intact plant to label the inner space sugars of internodal tissues. Tissue discs were cut, washed in running water, then incubated in solutions of unlabelled sucrose for 4 hours, after which the radioactivity of the external solution was determined. Previous analyses had shown that approximately 80 % of the label is contained in sucrose, glucose, and fructose under these conditions. Levels of sucrose above about 0.2 % do not increase the rate of accumulation (1). The results presented in table VIII show no significant effect of sucrose in the external medium on the efflux rate at concentrations up to 2.0 %, that is, ten times the saturating concentration for the accumulation reaction.

Movement of Sugars in Internodal Tissue of Defoliated Plant: The sugars of the fifth internode of a growing plant were labelled by feeding $C^{14}O_2$ to the fifth leaf and allowing photosynthesis and translocation to proceed for a 24-hour period. The leaves were then stripped from the stalk, and small tissue samples taken from the fifth internode at intervals over the next 48 hours. The stalk was protected from excessive evaporation by wrapping in a damp cloth. The results (cf. table IX) show that the sugars in the inner space of the intact plant are readily mobilized for utilization in growth, approximately 75 % of the sugar present at defoliation being removed in the 48-hour period after defoliation.

The initial rise in C^{14} content of the reducing sugars in the 6 hours after defoliation, together with the concomitant fall in the C^{14} content of sucrose, suggests that sucrose may act as a source of glucose

TABLE IX

CHANGES IN C^{14}-LABELLED SUGARS IN INTERNODAL TISSUE OF DEFOLIATED SUGAR CANE STALK

HOURS FROM TIME OF DEFOLIATION	C^{14} CONTENT dpm/mg FRESH WT				RATE OF LOSS OF SUCROSE dpm/mg FRESH WT/HR
	SUCROSE	GLUCOSE	FRUCTOSE	NON-SUGAR FRACTION	
Nil	109	59	53	91	
6	86	63	66	71	3.8
24	25	45	55	125	3.4
48	18	18	22	77	0.7

The fifth leaf of a growing cane stalk was fed 1.0 millicurie $C^{14}O_2$ for 10 minutes in full sunlight. After 24 hours, the stalk was defoliated and the spindle cut back to about three inches from the growing point, thereby preventing further photosynthesis and translocation from the leaves. A damp cloth was wrapped around the upper internodes to prevent excessive water loss. Tissue samples were removed from the middle region of internode 5, and the wound plugged with paraffin wax. Tissues were extracted in 3 volumes 96 % ethanol and the sugars assayed after chromatographic separation.

and fructose. The rate of loss of sugar over the first 24 hours is approximately seven milligrams per gram fresh weight/day, which is of the same order as the efflux rates for tissue discs in water. The rate of loss of sucrose was approximately constant over the first 24 hours. During the same period, the sucrose content declined by 77 %.

ANALYSIS OF SUGARS WASHED FROM INTERNODAL TISSUE DISCS INCUBATED IN C^{14}-LABELLED GLUCOSE: When C^{14}-labelled glucose is fed to tissue discs, 75 % or more of the C^{14} appearing in the sugars of the inner space is found in sucrose. Furthermore, when discs from immature internodes are placed in water, fructose leaks more readily than sucrose or glucose.

Taking advantage of these features, C^{14}-labelled tissue discs from the sixth internode were immersed for 4 hours in a solution of C^{14}-labelled glucose. The discs were then washed in water and the C^{14} content of the sugars in the wash water and in the tissue was determined (cf. table X). C^{14}-labelled sucrose was detected in the first washings and C^{14}-labelled fructose from the fourth washing onwards. Washing for 1 hour (which was the normal procedure adopted to remove outer space sugars) brought about a loss only of approximately 4 % of the sucrose in the tissue.

The results strongly suggest the sucrose washed from the tissue was derived from more than one pool as the C^{14} activity (dpm) in sucrose was washed from the tissue at the rate of 1,880/hour for the first 15 minutes of washing, 430/hour for the next 45 minutes and 260/hour for the last period of 75 minutes. The changes in rate could not be accounted for by the change in concentration gradient if it is assumed that the sucrose was contained in a single pool.

DISCUSSION

A number of criteria need to be satisfied in the formulation of a working hypothesis which will be in accord with present knowledge on the transformation and accumulation of fructose, glucose, and sucrose in sugar cane. These are:

TABLE X

C^{14} DISTRIBUTION IN SUGARS IN WASHINGS FROM TISSUE DISCS INCUBATED IN C^{14}-LABELLED GLUCOSE

SOLUTION ASSAYED	C^{14} CONTENT dpm $\times 10^{-5}$	% OF C^{14} ACTIVITY IN SUGARS			C^{14} CONTENT OF SUCROSE dpm
		SUCROSE	GLUCOSE	FRUCTOSE	
Initial medium	4.40	...	99	0.7	...
Medium after incubation	2.59	...	95	0.7	...
0–1 min wash water	0.86	0.25	73	0.7	210
1–2 min wash water	0.25	0.40	72	0.8	100
2–5 min wash water	0.076	0.80	70	0.7	60
5–15 min wash water	0.019	5.3	63	1.0	100
15–60 min wash water	0.012	27	41	3.5	324
60–135 min wash water	0.009	36	17	7.0	324
Ethanol extract of tissue	0.35	48	8.0	4.0	16,800

25 tissue discs from internode 6 were cut, washed for 1 hour in running tap water, then placed in 1.0 ml labelled glucose solution (3.7 μM/ml), and shaken for 4 hours. The ambient solution was withdrawn, 1.0 ml distilled water added to the tissue which was shaken gently for 1 minute, the solution then being withdrawn. This procedure was repeated for various time intervals over a 135-minute period, after which the tissue was extracted with 3 volumes 96 % ethanol. The sugars were separated using paper chromatography prior to assay for C^{14} activity.

I. Accumulation is an energy-requiring process, linked to respiration. In tissue slice experiments a rate-limiting step is observed, the kinetics corresponding to the Michaelis-Menten system for enzymes (1).

II. When tissue from young internodes is presented with radioactive glucose, 95 % of the sugar accumulated in the inner space appears as sucrose. The fructose moiety of sucrose is synthesized from the glucose supplied.

III. Although glucose supplied to tissue discs is stored as sucrose, it may be taken up at a rate ten times higher than sucrose similarly supplied.

IV. The net rate of movement of sugar is determined by the rates of two independent reactions, accumulation and efflux.

V. The rate of loss of sucrose from the inner space may be independent of the inner space sucrose concentration.

Certain mechanisms may be excluded as being inconsistent with the data. A rate-limiting carrier system at a permeability barrier which separates the outer space from the sites of sugar transformations is unlikely because:

A. To comply with Bieleski's observations on the competitive inhibition of sucrose uptake by glucose, the same carrier would need to transport both sugars, and hence the ratio of glucose to sucrose accumulation should be constant, under standard experimental conditions, whereas a threefold variation has been observed.

B. If the carrier was a diffusion carrier, the efflux rate should be sensitive to the presence of sugar in the external solution, which is also at variance with experimental results.

C. Inversion of sucrose in the outer space could account for Bieleski's data on competitive inhibition of sucrose uptake by glucose but would not account for results on relative rates of uptake of the sugars.

Sucrose synthesis is not likely to occur in the inner space because when young tissues are presented with radioactive glucose, 95 % of the sugar accumulated is sucrose and both hexose moieties are formed from the glucose supplied, with virtually no utilization of the large fructose pool in the inner space.

It follows that if the loci of sugar transformations are in the outer space (which is in diffusion equilibrium with the sugars in the external medium), the mechanism of transport of sucrose across the permeability barrier cannot be by combination with an activated carrier. This conclusion is based on the observation that glucose supplied in the medium may be accumulated as sucrose, but at a rate ten times higher than sucrose similarly supplied.

An alternative mechanism is based on the hypothesis that the sugars are activated prior to combination with a carrier. The sequence envisaged is:

Sugar + R–X ⟶ Sugar–X + R I

Sugar–X + carrier ⟶ Sugar-carrier + X .. II

Sugar-carrier (outer space) ⟶ Sugar (inner space) + carrier III

Sugar (outer space) ⟷ Sugar (inner space) IV

Sugar transformations, activation, and combination with the carrier are presumed to occur in the outer space, the carrier-sugar complex then discharging its sugar into the inner space. The net free energy change for equations I to III would be such as to allow accumulation against a considerable gradient. Efflux of sugar as described by equation IV is assumed to be a diffusion process. The data are as yet inadequate to suggest the nature of the activated sugars, the specificity of the carrier, or the location of the rate-limiting reaction for sugar accumulation in tissue discs.

It is reasonable to identify the inner space as the vacuole, and the sites of sugar transformations as being in the cytoplasm. The suggested mechanism has no bearing on the existence of other permeability barriers in the cytoplasm or which may separate the cytoplasm from the external solution. It implies only that such barriers are not detected with the techniques used.

Summary

I. When the source of radioactivity was $C^{14}O_2$ fed to a leaf attached to a sugar cane stalk, or C^{14}-labelled sucrose in a solution bathing internodal tissue discs, the pattern of labelling in the sugars in internodal tissue was similar.

II. The rates of accumulation of C^{14}-labelled sucrose and glucose into internodal tissue discs were compared. The rates for sucrose and glucose in tissues from very young internodes were 6 and 15 times higher, respectively, than for tissues from mature internodes. The ratio of the accumulation rates, glucose to sucrose, varied from 3.4 to 9.7. Regardless of whether sucrose or glucose was supplied in the medium, most of the C^{14} appearing in the sugars was found to be in sucrose.

III. Degradation of the sucrose accumulated into tissue discs from young internodes which had been incubated in a C^{14}-labelled glucose solution showed that the glucose and fructose moieties were approximately equally labelled. This tissue contained a large pool of unlabelled fructose, so that sucrose was synthesized at a locus which was not in rapid equilibrium with the fructose pool.

IV. When immature tissue discs were placed in distilled water, the relative proportions of the sugars (sucrose, glucose, and fructose) in the tissue were distinctly different from the sugars leaking into the medium. The results indicated that the immature tissues are eight to ten times more permeable to reducing sugars than to sucrose. Fructose was the major component of the reducing sugars leaking to the medium.

V. The rate of efflux of sugars from tissues in water is not closely correlated with the sugar content of the tissue. The rate first decreases then increases with increased maturity of the tissue.

VI. There was no significant effect of sucrose solutions at concentrations up to 2 % on the rate of efflux of sugars from tissue discs.

VII. $C^{14}O_2$ was fed to the fifth leaf of a stalk and the stalk was defoliated 24 hours later. Measurements of the mobilization of sugars in the fifth internode as indicated by the movement of the C^{14} showed that 75 % of the sugar was removed within 48 hours from defoliation. The rate of loss of sucrose was approximately constant over the first 24 hours following defoliation, although in the same period the sucrose content declined by 77 %. The results suggested that sucrose may act as a source of glucose and fructose. The rate of loss of sugar was of the same order as efflux from tissue discs in water.

VIII. When tissue which had accumulated sugar from a C^{14}-labelled glucose solution was transferred to water, labelled sucrose appeared in the water in less than a minute. Changes in the rate of efflux of sucrose indicated that there was more than one sucrose pool within the tissue.

IX. Several possible mechanisms to explain sugar transformations and accumulation are discussed. A working hypothesis is presented entailing a carrier which mediates the accumulation process and is located in a permeability barrier separating the outer and inner space of the tissue. The barrier is logically identifiable with the tonoplast membrane.

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PHYSIOLOGICAL EFFECTS OF GIBBERELLIC ACID. II. ON STARCH HYDROLYZING ENZYMES OF BARLEY ENDOSPERM [1]

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Two reports have appeared (15, 22) dealing with the effects of gibberellic acid (GA) on intact germinating barley. Both studies were carried out in an attempt to determine whether or not GA influenced the malting behavior of the barley, and extended the earlier observations of Hayashi (6) that GA speeded up germination and the various processes associated with germination, resulting in an enhancement of malt extract yield. GA, in both reports, markedly stimulated α-amylase activity and increased the growth of the leaf. Sandegren and Beling (22) came to the conclusion that the reduction in germination time (or, in other words, the hastening of starch hydrolysis, etc.) was due to GA's effect on embryo growth.

The first paper of this series (19) presented evidence of the production of reducing sugars and an increase in amylase activity resulting from the treatment of barley endosperm with GA. Although it has not yet been possible to demonstrate a direct relationship between increased amylase activity and the production of reducing sugars in the intact grain, several results, such as anion and heat effects, and the increased liberation of maltose, lend circumstantial support to the importance of the role of amylase activity in starch hydrolysis in the isolated endosperm.

These results also lead to the suggestion that an endogenous gibberellin, such as that present in germinating barley (21), might, in fact, play an important hormonal role during the germination process, and prompted a closer investigation of the changes in starch hydrolyzing activity of solutions obtained from water, and GA-treated barley endosperm.

It should be borne in mind that the results discussed here were obtained with barley endosperm alone, i.e., after the embryo had been removed.

MATERIALS AND METHODS

Seeds of *Hordeum distichon* L. var. Prior, of the 1957 harvest were used. All water used was doubly glass distilled and all GA solutions were freshly prepared for each experiment.

The seeds were stored and sterilized exactly as described earlier (19). Endosperm free from embryo tissue were obtained after sterilization by cutting the grain in half transversely, and were placed into 5 mm petri dishes. The ambient solution in the petri dishes contained 0.3 ml 0.01 M acetate buffer (pH 5.0) and 0.05 ml streptomycin (500 γ). To this mixture either 2.65 ml water or 2.65 ml solution containing 200 γ GA (final concentration was 2×10^{-4} M) was added, bringing the total volume to 3 ml. The dishes were kept at 24° C for 22 hr.

After incubation the solutions from the petri dishes of GA-treated endosperm were combined as were the solutions from the water-treated endosperm, and gently mixed so as to avoid any frothing which greatly decreases enzyme activity. An aliquot of each was taken for protein nitrogen estimations and the remainder was assayed, treated, or frozen immediately. These solutions were used as the enzyme and all procedures were executed identically with both solutions.

Amylase assays contained 0.05 ml streptomycin (500 γ), 0.5 ml 0.01 M acetate buffer (pH 4.6), 2 ml 0.87 % soluble potato starch (B.D.H., A.R.), water, and enzyme (either control or GA-treated) to a volume of 5 ml. Enzyme and starch controls were also run and the results were subtracted from the test assays. The assays were carried out at 30° C and cotton-stoppered pipettes were used as a precautionary measure. At the indicated times, 0.2 ml aliquots of the assays were taken and added to 0.2 ml 4×10^{-4} M mercuric chloride and 0.1 ml IKI solution (0.2 % iodine in 2 % potassium iodine). This was immediately mixed and diluted to 10 ml with water. The choice of 0.1 ml IKI for these experiments was based on the fact that the absorbance produced by this amount of reagent in the absence of starch is only 1 % of that obtained in the presence of the maximum amount of starch employed. The aliquot of starch taken (0.2 ml) produces an absorbance which lies within the proportionality range of the reagent and yet leaves a large scope for determining hydrolytic losses due to enzyme action. Absorbances were determined after 25 minutes, at 600 mμ on a Unicam SP 350 spectrophotometer.

Aliquots (0.05 ml) of the assays were also taken at the stated times for reducing sugar determination. [Although Shaffer and Somogyi (23) stated that starch interferes with the reducing sugar reaction, it was found that at the dilution employed in these experiments it exerted no effect at all.] The aliquots were added to 0.2 ml 4×10^{-4} M mercuric chloride and 0.75 ml water and immediately mixed, after which the Somogyi copper reagent was added, as outlined previously (18).

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The inactivation of α-amylase was accomplished by subjecting the solutions to pH 3.3 (at 0° C) for 24 hours as recommended by Hopkins et al (8) and not for only 15 minutes as is usually done (17). Portions of the enzyme solutions were brought to room temperature and the amount of 0.1 N hydrochloric acid needed to lower their pH to 3.3 was determined. The amount of 0.1 N NaOH then required to raise their pH to 4.6 was also determined. Similar amounts of acid and base were added to the enzyme solutions at 0° C before and after incubation for 24 hours and account was taken of the enzyme dilution thereby incurred so as to ensure that the same amount of enzyme solution (0.5 ml of original extract) was present in all assays.

Formamide, which has been used to differentiate between the two amylases (9), was found to completely obscure the reducing sugar test and was therefore not further investigated.

The inactivation of β-amylase was accomplished by holding the enzyme solutions at 70° C for 15 minutes. Identical results were obtained by heating the solutions for only 10 minutes. Aliquots (1 ml) of the enzyme solutions were added to 1 ml of 0.01 M calcium acetate. They were then placed in a water bath at 70° C for exactly 15 minutes after which they were plunged into ice water. The method is essentially that of Kneen, Sandstedt, and Hollenbeck (12). For the purposes of this work, utilization of the widely-accepted ASBC (1) procedure for determining α-amylase activity would have made it highly likely that erroneous conclusions would be drawn from the results.

Protein nitrogen estimations were made according to the technique described by Lowry (13).

Results

The experiments reported below were all performed on the same preparation so as to enable a more accurate comparison to be made. An analysis of the amylolytic activity of enzyme solutions obtained from control and GA-treated endosperm before preferential inactivation treatments were imposed, is represented in figure 1. The shaded symbols were obtained with enzyme solution from GA-treated endosperm and the open symbols represent the activity of the water-control enzyme solution. The squares are indicative of the percentage loss in IKI-starch absorbance values while the circles illustrate the apparent percent conversion of the starch into anhydrous maltose (16). Because potato starch, the substrate used in these experiments, consists of both amylose and amylopectin, β-amylase acting alone is able to convert only 60 % of it into maltose and cause only a 60 % loss in IKI-starch absorbance. If other enzymes capable of hydrolyzing starch are also present, the hydrolysis limit exceeds 60 %. From an examination of the water-control curves of figure 1, it may be seen that at the end of 21 hours the conversion to maltose almost reached 60 % while the loss in IKI-starch absorption just exceeded 60 %. This indicates that there was no, or only the barest trace of, enzyme capable of hydrolyzing starch, other than β-amylase, present in the assay. On the other hand, with the enzyme obtained from GA-treated endosperm, there was a greater than 60 % conversion to maltose and a greater than 60 % loss in IKI-starch absorbance within 1 hour.

Figure 2 presents the results obtained when the enzyme solutions were subjected to heat treatment. The water control enzyme retained just the faintest indication of hydrolytic capacity whereas the solution from GA-treated endosperm was still capable of causing a fairly rapid hydrolysis of starch. It is of interest to note that there was a much faster loss in IKI-starch absorbance than apparent maltose production. This is indicative of α-amylolysis and is in keeping with the concept that the treatment differentially inactivates β-amylase leaving at least 93 % of the α-amylase activity functional (5, 20).

A shift in the wavelength at which the IKI-starch reaction exhibits maximum absorption is also characteristic of α-amylase activity in the hydrolysis of starch. During the course of hydrolysis a change in the wavelength of maximum absorption was noted, with the peak falling to 550 mμ at the time of about 90 % hydrolysis (as determined at 600 mμ).

Alpha-amylolysis can also be characterized by plotting the percent loss in IKI-starch absorbance against the percent increase in apparent maltose. This has been done for the results obtained after heat treatment (fig 3). The curve follows exactly what might be expected (24) of α-amylase action and leaves no doubt that treatment of barley endosperm with GA caused the liberation of an appreciable amount of α-amylase. This is, as far as is known, the first demonstration that α-amylase is present in appreci-

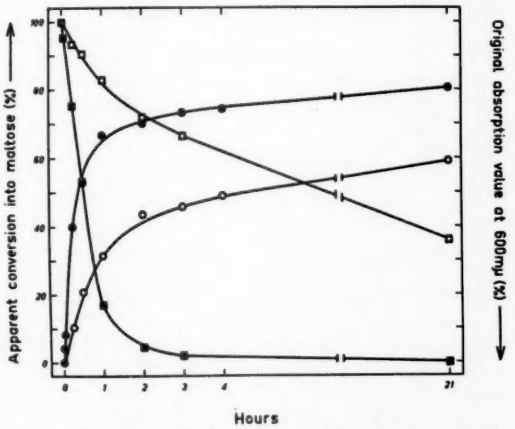
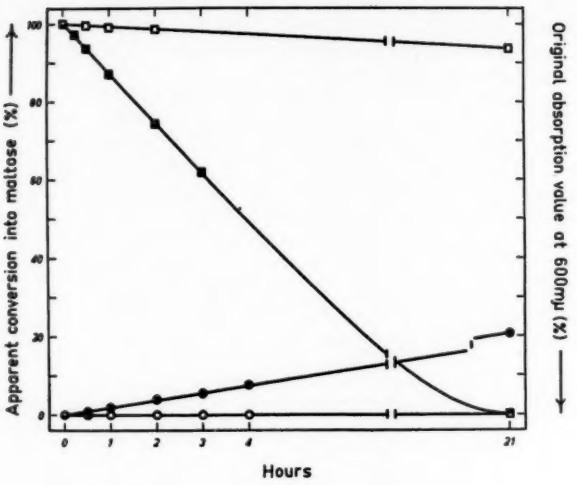
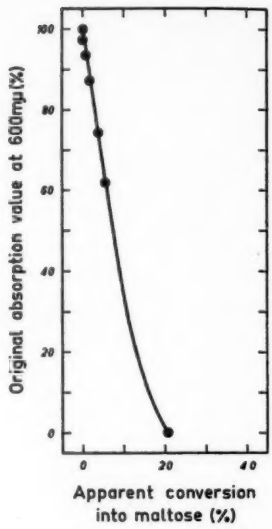
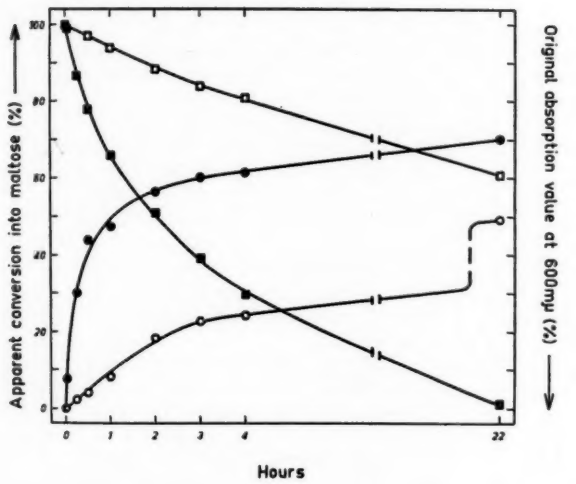


FIG. 1. Starch hydrolyzing activity of enzyme obtained from GA-treated (shaded symbols) and water-treated (open symbols) endosperm. Circles illustrate percent apparent conversion of starch to maltose; squares indicate percent loss in absorbance of IKI-starch reaction.







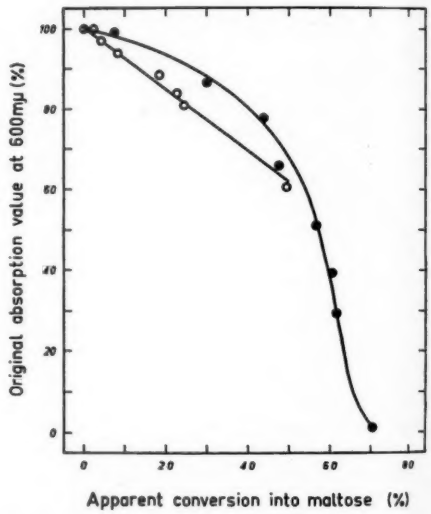


FIG. 2 (*top left*). Starch hydrolyzing activity of GA-treated (shaded symbols) and water-treated (open symbols) endosperm extracts after treatment 70° C for 15 minutes in the presence of Ca^{++}. Circles illustrate percent apparent conversion of starch to maltose; squares indicate percent loss in absorbance of IKI-starch reaction.

FIG. 3 (*top right*). Absorbance-conversion curve illustrating amylolytic activity after treatment at 70° C for 15 minutes of endosperm extract obtained by GA application.

FIG. 4 (*bottom left*). Starch hydrolyzing activity of enzyme obtained from GA-treated (shaded symbols) and water-treated (open symbols) endosperm after 24 hours at 0° C and pH 3.3. Circles illustrate percent apparent conversion of starch to maltose; squares indicate percent loss in absorbance of IKI-starch reaction.

FIG. 5 (*bottom right*). Absorbance-conversion curves of GA-treated (●) and water-treated (○) endosperm extracts following treatment for 24 hours at 0° C and pH 3.3.

able amount in, and can be extracted from, barley endosperm without the mediation of embryo action.

Effects of acid treatment (to remove α-amylase) on amylolytic activity are not as precise as the effect of heat treatment, since small, varying amounts of β-amylase may also be inactivated. However, the technique is efficient in rendering α-amylase inoperative, particularly when applied for a 24 hour period (8). The results obtained with the two enzyme solutions following pH treatment are presented in figure 4. The water control curves did not reach the 60 % hydrolysis limit by the time the experiment was terminated (22 hr). On the other hand, the GA-extracted enzyme retained the capacity to reach and exceed 60 % hydrolysis by the end of 4 hours.

Results almost identical with those shown in figure 4 were also obtained by subjecting the enzyme solutions to a treatment of 25° C at pH 3.3 for a half hour. The combination of low pH and higher temperature has also been shown to completely eliminate α-amylase activity (8). If the technique used actually did inactivate all of the α-amylase present (8, 17) then we must conclude that in the GA-extracted solution there is some other enzyme also present, which, in conjunction with β-amylase, is capable of causing more than 60 % hydrolysis of the starch substrate.

Figure 5 indicates the types of curves obtained when the activities of the enzyme solutions are plotted as for figure 3. The curve for the activity of the water control is similar to what might be expected (24) for β-amylolytic action, whereas the activity curve of the enzyme obtained from GA-treated endosperm exhibits characteristics of mixed enzyme action.

The identity of the third enzyme, whose action appears to be similar to that of α-amylase, is still in doubt, although it may be R-enzyme which has been shown to withstand the pH treatment (14). It is interesting that had the ASBC (1) method of analysis for α-amylase been used, the conclusions drawn from the experiments would have been substantially different. The results would have been interpreted as an excessive stimulation of α-amylase activity by GA treatment, since the test makes no provision for the possible contribution of other amylolytic enzymes.

The protein nitrogen content of the ambient solutions [as estimated by the Lowry test (13)] was 22 γ N/ml in the absence, and 37 γ N/ml in the presence of GA. These values indicate that the endosperm treated with GA liberated considerably more protein than did the water-treated endosperm. The increased amount of protein liberated by the GA-treated endosperm may account for the increased enzyme activities observed.

On the basis of eight endosperm per 3 ml solution per petri dish, the enzyme activities in each 0.5 ml aliquot measured, represent the contribution of only 1.3 endosperm.

Discussion and Conclusions

The importance of amylase action during germination has been questioned (24) and although these results and those of the previous paper of this series (19) present evidence for the activity of amylase during the hydrolysis of starch, there is no reason to limit the responsibility of starch degradation to one enzyme system. Certainly other enzymes, as well as the amylases are present and active in the endosperm that has been treated with GA, and the breakdown of starch, as it occurs in vivo, may be the result of the action of several, if not many enzymes. The analysis of sugar liberated during GA treatment indicated the production of more glucose than could be explained by amylase alone (19), and tends to support the concept of multi-enzyme action on starch.

As recently as 1958 suggestions have appeared in the literature to the effect that β-amylase activity in the endosperm during germination is a function of embryo secretion (11). This has been shown to be unnecessary as an explanation of the increase in β-amylase activity which occurs. Alpha-amylase has also been thought of as an embryonic exoenzyme, with possibly more reason than β-amylase. There is no, or almost no, α-amylase activity in the resting grain. Extracting the resting grain with saline solutions, or treating it with such things as Ca^{++}, sulfhydryl compounds, proteolytic enzymes, etc., all of which increase the extractable β-amylase, does not cause more than a nominal increase in the amount of α-amylase extracted. As a result it was reasonable to suppose that α-amylase activity during germination reflected enzyme synthesis (which would occur in the embryo) followed by secretion of the enzyme into the endosperm.

The present experiments, however, demonstrate that α-amylase, like β-amylase, is already present in the endosperm, as are other amylolytic enzymes, and indicate that other than direct embryo action, GA is the only agent yet known which is capable of causing an activation of these enzymes. It is reasonable to suppose, therefore, that the in vivo activation of these enzymes results from the action of an endogenous gibberellin which is produced in the embryo during germination and liberated into the endosperm.

The nature of the amylases seems to be in doubt. Danielsson and Sandegren (4) and Ayrapaa and Nihlen (2) have indicated that both α-, and β-amylase of malt are albumins. On the other hand, Hopkins (7) and Wiener and Hopkins (25) have suggested that the amylases are more like globulins in respect to their increased solubility in salt solutions. Whether or not α-amylase acts as an albumin or globulin following the malting treatment, it acts as neither before malting. GA, although not capable of increasing the activity of amylase molecules, (6, 19), appears to have the capacity, like embryo action, of causing the inactive, presumably insoluble protein to become both soluble and active.

A situation which may be comparable is that described by Ikuma and Thimann (10) in which lettuce seeds, having a red-light requirement for germination that can be circumvented by GA treatment, achieve germination by enzyme activation. Granting Brian's suggestion (3) that red light results in the formation

of a GA-like substance, it is very possible that this hormone activates Ikuma and Thimann's enzyme in the same way that GA activates the amylolytic system in the barley endosperm.

Summary

The activation of amylolytic enzymes in the barley endosperm as a result of GA treatment has been investigated. A substantial increase in β-amylase activity was noted as well as the initiation of α-amylase activity. A third enzyme, similar in aspects to α-amylase, is also present as a response to GA and is capable of causing, in conjunction with β-amylase, the hydrolysis of more than 60 % of the starch present in the amylolytic assays. These activities are in marked contrast to the amylolytic activity of control treatments. Since GA application to the excised endosperm results in the liberation of simple carbohydrates and enzymes which are capable of causing the formation of such sugars, it is suggested that the control of carbohydrate metabolism in the barley endosperm during germination may be a function of an endogenous embryonic gibberellin.

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METABOLIC AND NON-METABOLIC UPTAKE OF SODIUM IN ROOTS OF ZEA MAYS[1,2]

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INTRODUCTION

It has been recognized for many years that part of the ions that enter plant tissue bathed in a salt solution are not irreversibly sequestered but enter and are free to leave again in accordance with Fick's law of diffusion and principles pertaining to Donnan or ionic exchange equilibria (8, 9, 13, 16). This phenomenon is generally ascribed to the non-metabolic diffusive movement of salt into a part of the tissue (the free space) which presents little if any resistance to the entry of dissolved materials. Some importance has been attached to the measurement of free space in terms of the relative volume of tissue involved. The difficulties inherent in such measurements have been discussed thoroughly by Briggs and Robertson (3) and need not be considered here. In this article the authors have followed Jacobson et al (13) and have sought to avoid questions of volume and space by using the term "non-metabolic uptake". Arbitrarily we have included under this heading ion uptake at low temperatures, in an atmosphere of N_2, or in the presence of metabolic inhibitors.

Studies of non-metabolic absorption have been carried out with tissue slices and whole roots (16). Most of the tissue comprising these materials consists of mature cells since even where whole roots are used the meristematic portion amounts to only a small fraction of the sample taken. Over and above their capacity for non-metabolic uptake of salt such tissues exhibit a pronounced ability to absorb ions metabolically by a process that is relatively irreversible. This phenomenon makes necessary the postulation of a barrier either within the cell or at its surface which is impermeable to ions in the medium. According to some evidence (1), the barrier is perhaps the tonoplast, the vacuoles representing a sink or dead end in which ions are trapped as they move through the symplast. The possibility is not ruled out that the plasmalemma may represent the barrier, but if this were the case, it would become necessary to limit non-metabolic uptake of ions to the cell walls and the intercellular spaces (16, 17). Yet a third conception is that the whole cytoplasm constitutes a barrier, that is, there may be ionic exchange sites throughout the cytoplasm which are but slowly accessible to ions of the medium (19). In this view ultimate secretion of ions into the vacuole may result from a breakdown of protoplasmic structure.

Because of these general questions studies of ion absorption in immature cells are of interest, in particular cells in which the vacuole is either absent or at least minute and possibly not yet delimited by a tonoplast. For example, if the tonoplast constitutes the barrier, then such tissues should exhibit no ability for the metabolic accumulation of ions.

Root tips of maize proved to be excellent for this purpose. Microscopic examination indicates that the cells extending at least 1.8 mm from the tip are essentially non-vacuolated, although occasional cells are seen in which extremely small structures resembling vacuoles may be found. Most of the cells in this region display mitotic figures when suitably stained. The chief purpose of this work was to study the absorption characteristics of these undifferentiated cells and to compare them with those farther from the tip in which vacuolation is progressively more nearly complete.

EXPERIMENTS

Dark-grown (26° C) tips of maize roots (Peoria variety) were used in this investigation. The seed was soaked in aerated distilled water for 24 hours and then spread on cheese cloth supported by a well tinned screen. The screen was then supported in a pyrex baking dish filled with 0.25×10^{-3} M $CaCl_2$, the cheese cloth dipping into the solution. Another baking dish was inverted over the first to maintain a humid atmosphere. The roots were harvested on the 4th day.

After excision, the roots were blotted dry and the terminal portions were cut into segments, 0 to 1.8 mm, 1.8 to 3.8 mm, 3.8 to 7.8 mm, and 7.8 to 11.8 mm measured from the tip. A simple cutting device composed of four thin razor blades bolted to two machine screws and spaced with washers permitted sectioning of twenty roots simultaneously; a block of paraffine was used as a cutting board. Forty of each of the segments were used for each experiment after the fresh weights had been determined.

Microscopic examination of the segments indicated that physical cell damage occasioned by cutting was negligible. In a preliminary experiment absorption and retention of Na by sectioned and unsectioned root tips 11.8 mm long were studied using labeled NaCl.

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[2] This paper is based on work performed under Contract No. AT-(11-1)-34, Project 5, with the U.S. Atomic Energy Commission.

[3] Present address: Instituto Politecnico Espanol, Tangier, Morocco.

In 10 hours 40 root tips sectioned as described above absorbed 15.0 μe of Na whereas the value for unsectioned tissue was 14.0 μe. After 10 hours in non-labeled 0.005 M NaCl at 0° C, both sectioned and unsectioned root tips were found to have retained 73 % of the sodium previously taken up. Thus, sectioning had an appreciable effect neither upon the ability of this material to take up Na nor upon its ability to retain Na against isotopic exchange.

The initial phase of this research was devoted to studying Na uptake by the segments as influenced by the concentration of NaCl in the medium. For each uptake determination, a weighed batch of 40 segments was placed in 500 ml of the appropriate NaCl solution for 8 hours at 21° C. The initial pH of the culture solution was 6.0. At the end of the absorption period the segments were washed twice in 500 ml of distilled water for about five minutes and then filtered out each time on a nylon net. The washed segments were ashed and analyzed for Na by a Beckmann flame photometer with photomultiplier attachment. Each quantity plotted on the ordinate (fig 5) represents an average of the uptake values for three separate batches of 40 segments.

The second research phase dealt with metabolic and non-metabolic uptake of sodium by the root segments. The experiments were carried out with Na^{22} (half-life, 2.6 yr). Using NaCl solutions labeled with this isotope, it was possible to determine time curves on single batches of 40 segments. In these studies, however, the segments were not washed in distilled water after an absorption period. Instead, the segments were blotted free of adhering liquid and the sodium content of the fresh tissue was determined by counting the Na^{22} gamma emission with Berkeley Decimal Scaler (Model 2001) and a lead shielded scintillation detector. Since the blotting and counting of the segments were carried out in less than 10 minutes, the segments could be returned to the culture solution for another absorption period. While being counted the tissue was sealed in the planchet to avoid drying out.

This procedure was used to determine the sodium uptake versus time curves for the various sections of maize roots at 2° C and 26° C. For each curve 40 segments of tissue were placed in 500 ml of 0.005 N NaCl labeled with 0.02 μc of Na^{22} per ml. The initial pH of the culture solution was 6.0; the pH did not vary appreciably throughout the experiment. The curves for 2° C and 26° C are presented in figure 1.

Experiments conducted in this way with different batches of roots were found to give consistent results as evidenced by the data of table I. Although only the 24 hour absorption data are presented, the data obtained for other absorption periods were similarly consistent.

The Na^{22} was also used in two isotopic exchange studies with the various root sections. These experiments were suggested by the general observation that isotopic exchange between ions of the culture medium and metabolically absorbed ions usually is slow whereas isotopic exchange between ions of the medium and non-metabolically absorbed ions often is rapid. In the first study 40 of each of the four segments were immersed for 11 hours at 26° C in 500 ml of a 0.005 N NaCl solution labeled with 0.02 μc of Na^{22} per ml. Then the sections were removed, blotted dry, and their radioactivity measured. Each batch of 40 segments was then placed in unlabeled 0.005 N NaCl at 2° C. The loss of Na^{22} by the segments with time was determined by removing the segments periodically and counting them as previously described (fig 3). In the second study, batches of the various segments each were first placed in 10 ml of 0.010 N NaCl labeled with 0.10 μc of Na^{22} per ml for 2 hours at 26° C. They were then removed, blotted dry, and their radioactivity was determined. Following this, one batch of each segment was placed directly in 10 ml of unlabeled 0.010 N NaCl at 0° C. The other batches were so treated after having been allowed to stand for periods up to 3 hours in a moist chamber at room temperature (~21° C). After 2 hours in cold NaCl solution the tissue was again blotted and counted, and the retained percentage of Na^{22} was calculated (fig 4).

Total nitrogen determinations were made on the root tissue by wet digestion with sulfuric acid followed by distillation of the ammonia using a micro-Kjeldahl still. The distilled ammonia was determined by titration with HCl. The total nitrogen content and the percentage of dry matter found for the various segments are shown in table II.

The water was laboratory distilled water further purified by passage over a Dowex-50 resin column.

TABLE I

24 HOUR UPTAKE OF SODIUM FROM 0.005 N NaCl

SEGMENT	NA UPTAKE, INDIVIDUAL VALUES, MEQ/KILO, WET WT	NA UPTAKE, MEAN, MEQ/KILO	STANDARD DEVIATION, σ*	CONFIDENCE LIMITS, 95 %*
0–1.8 mm	61.0, 48.5, 50.1	53.2	6.80	36.3– 70.1
1.8–3.8 mm	122.6, 126.2, 119.3	122.7	3.45	114.1–131.3
3.8–7.8 mm	92.4, 96.5, 97.8	95.6	3.98	85.7–105.5
7.8–11.8 mm	70.5, 77.8, 72.8	73.7	3.72	64.4– 82.9

* Terminology of Brownlee (6).

Fig. 1

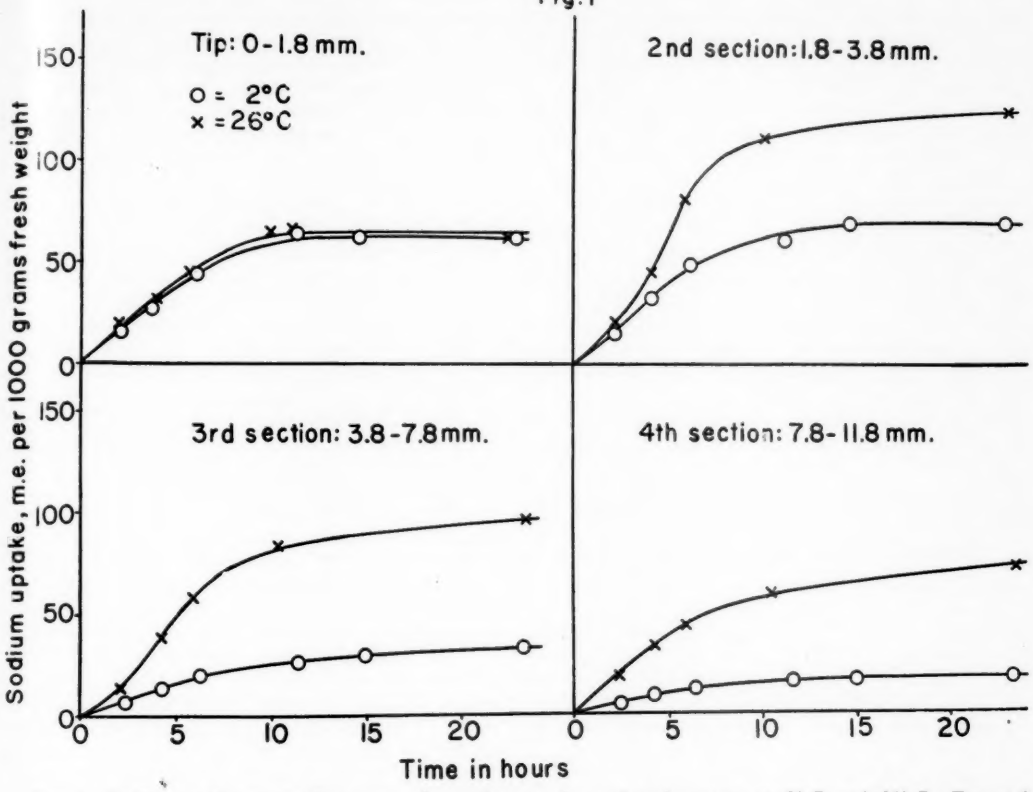


FIG. 1. Sodium uptake versus time curves for various sections of maize roots at 2° C and 26° C. For each curve, 40 segments of tissue were placed in 500 ml of 0.005 N NaCl labeled with 0.02 μc of Na^{22} per ml. The pH of the culture solution was 6.0. At the intervals indicated in each curve, the segments were removed briefly from the medium, blotted dry, and counted. The uptake of sodium as meq/1,000 g fresh wt was then calculated using the known fresh weight of the segments.

DISCUSSION

The data of table II are in accord with those obtained by Brown and Cartwright (7) for root tips of maize. Although no respiratory measurements have yet been made here, it is reasonable to suppose on the basis of data obtained elsewhere that the first segment represents a region of relatively rapid metabolism. Goddard and Meeuse (10) have found the most active respiration to occur in the apical millimeter of corn roots. The data of Brown and Broadbent (4) and of Lund, Vatter, and Hanson (18) indicate that regions of the root of pea and corn, respectively, which are highest in dry matter and protein nitrogen also respire most actively.

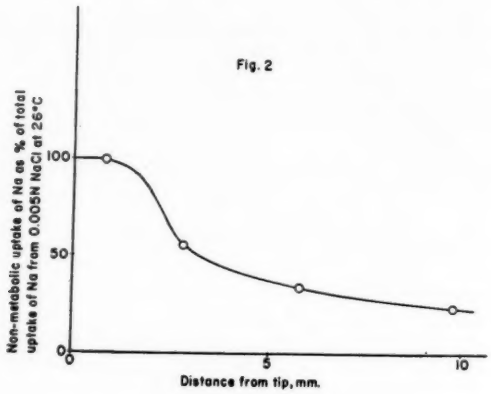
TABLE II

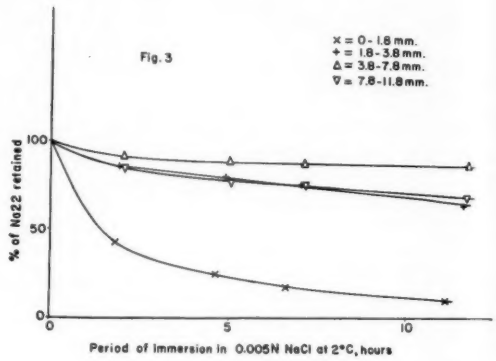
NITROGEN AND DRY MATTER CONTENTS OF VARIOUS SEGMENTS OF MAIZE ROOTS

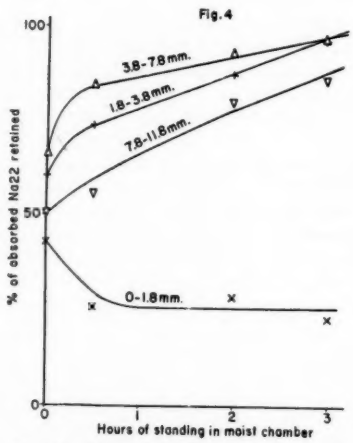
SEGMENT	NITROGEN, MG/G, WET WT	DRY MATTER % WET WT	NITROGEN % DRY WT
0–1.8 mm	14.2	15.2	9.3
1.8–3.8 mm	5.9	8.3	7.1
3.8–7.8 mm	2.6	6.3	4.1
7.8–11.8 mm	2.0	5.6	3.6

The presumed high metabolic activity of the first segment is, however, not associated with a parallel high rate of sodium uptake (fig 1). This apparent anomaly has been previously noted in studies dealing with the absorption of potassium by maize roots (5). As with K absorption, the first segment shows a relatively low rate of sodium uptake. Kramer and Wiebe (12) on the other hand report that phosphate is usually more actively absorbed by cells at the root tip than by those immediately behind it in the region of elongation. Of greater significance perhaps is the form of the time curve of absorption for the first segment (fig 1). Absorption by this segment has apparently

ceased after about ten hours exposure to the solution whereas in the case of the more mature segments the process has gone on for at least 24 hours although at a reduced rate. There are at least two possible reasons for this behavior. The obvious possibility is that the rapidly respiring first segment has completely exhausted its reserves of substrate after ten hours and therefore is unable any longer to provide energy for active accumulation although the barrier is still functional and preventing any appreciable loss to the surrounding medium. A second possibility is that absorption by the first segment is wholly or mostly non-metabolic and that the immature cells comprising this segment are incapable of irreversibly absorbing







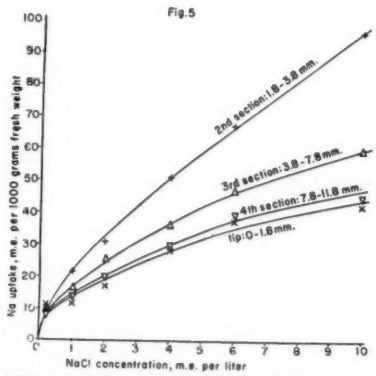


FIG. 2. Curve showing the importance of non-metabolic Na absorption as a function of distance from the tip in maize roots. The curve was derived from the data of figure 1 assuming that the uptake at 2° C is entirely non-metabolic. The values on the ordinate were obtained by dividing the Na uptake for 24 hours in 0.005 N NaCl at 2° C by the uptake for 24 hours in 0.005 N NaCl at 26° C.

FIG. 3. Curves showing the percentage of absorbed Na^{22} retained by maize root segments after various periods of immersion in 0.005 N NaCl at 2° C. Prior to the experiment, the segments were placed for 11 hours at 26° C in a 0.005 N NaCl solution labeled with 0.02 μc of Na^{22} per ml.

FIG. 4. Graph showing percentage of absorbed Na^{22} retained by maize segments against a 2 hour immersion in 0.01 N NaCl at 0° C following a period of standing in a moist atmosphere at 21° C. The Na^{22} was absorbed initially as the result of an immersion of the segments in 0.01 N NaCl labeled with 0.10 μc of Na^{22} per ml for 2 hours at 26° C.

FIG. 5. Na uptake versus concentration curves for various segments of maize roots. For each uptake determination, 40 segments were placed in 500 ml of the appropriate NaCl solution for 8 hours at 21° C. Each quantity plotted on the ordinate represents an average of the uptake values for three separate batches of 40 segments. The initial pH of the culture solutions was 6.0.

sodium. If this is the case we must assume that after about ten hours the tissue of the first segment has arrived at equilibrium with respect to the distribution of sodium between itself and the solution. The experimental results reported in figure 3 support the second possibility. At the end of the experiment less than 10 % of the original activity remained in the first segment whereas about 88 % had been retained by the third segment and 70 % by the fourth. The behavior of the second segment requires some comment. While being much more retentive than the first segment, some 65 % of the initial activity remaining after 11 hours, the second segment is not in equilibrium with the medium at the end of the experiment but is continuing to give up sodium. The reason for this is not clear but it seems most probable that this phenomenon is related to the presence of non-vacuolated tissue in the second segment. Repetitions of this experiment yielded essentially the same results.

From figure 1, where the absorption versus time curves for 2° C and 26° C are compared, it is apparent that metabolism plays a minor role in absorption of sodium by the first segment whereas in succeeding segments its part is considerable and increasingly so as the cells mature. In this connection, it is interesting that the increase in cell wall material and intercellular space taking place as the cells mature does not lead to an increase in the capacity for non-metabolic sodium absorption, but quite the reverse. This fact appears to indicate that something more than the outer reaches of the cell is involved in this type of uptake. Also, the relatively long period required for the first segment to come to equilibrium with the environment suggests strongly that the essentially non-metabolic uptake of sodium involves an attachment of the ion to sites within the protoplasm and perhaps an incorporation of sodium in protoplasmic structure as has been suggested elsewhere (19). If this latter idea is correct we must suppose, however, that a much lower level of metabolic activity is required for such incorporation than is needed for accumulation in vacuoles.

The uptake versus concentration curves of figure 5 are interesting in that the curve for the first segment does not appear to be different in kind from the curves for the other segments. For example, the curves for the first and fourth segments are quite similar even though a profound difference in the character of the uptake is indicated by other data presented in the paper.

Figure 2 was derived from the data of figure 1 by dividing the Na uptake value obtained after 24 hours at 2° C by that obtained after 24 hours at 26° C in 0.005 N labeled NaCl to obtain an estimate of the percentage of the total absorption which is non-metabolic in each of the four segments. Of course, the percentages obtained apply only to the particular concentration of sodium employed. The result indicates a rapid decline in the importance of non-metabolic uptake over the first 3 mm. Farther back the rate of decline is progressively slower. The form of the curve indicates that the contribution of non-metabolic absorption may become fairly constant beyond 11 mm or so from the tip.

The ability of the various segments to transform non-metabolically absorbed sodium into a more difficultly exchangeable form while standing in a moist chamber at 21° C is depicted in the curves of figure 4. The results indicate that the first segment possesses no ability for this presumably metabolic transformation whereas the older tissues show considerable ability in this regard. Indeed, as a result of standing in the moist chamber the first segment showed a decrease in the retention of absorbed Na^{22} against the action of an eluting NaCl solution. Quite possibly the decrease was due to some loss of sodium by the first segments to the slightly damp filter paper used to support them in the moist chamber. Such losses, of course, may have occurred with the older segments but were more than counterbalanced by metabolic accumulation.

The experimental results indicate that meristematic tissue of maize roots possesses little if any ability to absorb sodium metabolically, but that this ability develops rather rapidly during maturation. Whether or not this is true for other cations or for anions has not been determined. The concurrence of this development with any other phase of maturation is not proven by these experiments. However, the development of vacuoles with well defined tonoplasts does seem to coincide and it is at least reasonable to believe that the connection may be causative and that the possibility of secretion to vacuoles is a prerequisite for metabolic uptake.

The question as to the location of the barrier region operative in metabolic absorption cannot be answered definitely on the basis of this research. Because of the evidence that at least part of the cytoplasm is involved in non-metabolic uptake, however, it may be presumed that the barrier is situated deeper in the cell than the plasmalemma. The writers are inclined to believe that the cytoplasm itself is rather resistant to the entry of sodium and probably to other ions and may actually constitute the barrier region.

Summary

Using separate sections of the root tips of maize and NaCl solutions labeled with Na^{22}, the uptake of sodium by essentially non-vacuolated tissue (0-1.8 mm from tip) has been compared with that in tissues farther from the tip in which vacuolation is progressively more nearly complete.

For the non-vacuolated tissue, the sodium uptake versus time curves at 2° C and 26° C were nearly identical. In the older tissues, however, a much greater sodium uptake was observed at 26° C than at 2° C.

Isotopic dilution experiments with Na^{22} showed in the case of the non-vacuolated tissue an almost complete isotopic equilibrium between sodium of the tissue and sodium of the medium after a period of

about ten hours whereas the vacuolated tissues were still far removed from isotopic equilibrium.

By means of Na^{22} it was found that vacuolated tissues are able to transform non-metabolically absorbed sodium into a more difficultly exchangeable form during standing at 21° C in a moist chamber. Non-vacuolated tissue is unable to accomplish this.

On the basis of the experiments it is concluded that sodium uptake in the non-vacuolated sections is purely non-metabolic. That is, the ability for the metabolic absorption of sodium appears to coincide with the development of well defined vacuoles.

Because of the relatively long time required for isotopic equilibrium between sodium of non-vacuolated tissue and sodium in the culture medium, it is concluded that at least part of the cytoplasm is involved in the non-metabolic uptake of that element.

The sodium uptake versus concentration curves were of essentially the same form for the various root sections. This was the case in spite of the profound differences in the character of sodium absorption in tissues at different stages of development.

Acknowledgment

The authors are greatly indebted to Mr. D. Branton for advice and assistance in preparing the root tissue for microscopic examination.

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RELATIVE UPTAKE AND TRANSLOCATION OF POTASSIUM AND CESIUM IN BARLEY[1, 2]

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INTRODUCTION

The ability of plants to absorb ions selectively is a major reason for the preference for chemical rather than physical theories of ion uptake (11), yet in spite of this, comparatively little attention has been paid to this property. Thus far, selectivity between alkali cations has been examined in excised barley roots (6) in beet discs (12) and in whole plants (1, 4).

In recent years the relative absorption of potassium and cesium has become of practical interest because of the position of Cs^{137} as one of the principal long-lived fission products from nuclear weapons. While cesium behaves in a qualitatively similar fashion to potassium in absorption by excised roots (2, 6) and by whole plants (4, 9) and also in redestribution through plants (10), simultaneous determinations of the rates at which potassium and cesium are absorbed have rarely been made.

The experiments reported here were designed to examine the relative rates at which cesium and potassium were absorbed by roots and were transferred to the shoots of young barley plants. It was desired in particular to examine the effect of varying the external concentration of potassium. It was therefore not possible to grow plants for long periods of time since the experimental treatments would lead to differences in growth rate. Further, the interpretation of results over long periods may be complicated by differential redistribution of potassium and cesium during growth. For these reasons a short experimental period was adopted.

From a practical point of view interest lies in the behavior of carrier-free Cs^{137} rather than of macro amounts of cesium. However, it is not legitimate to compare the absorption of Cs^{137} and stable potassium over short periods since, whereas a gross uptake of the radioactive Cs^{137} is observed, the uptake of stable potassium is measured as a net result of an influx and an efflux of the element into and out of the tissue. In the extreme case, no uptake of stable potassium might be measured whereas a radioactive isotope of potassium might be taken up to a considerable degree by exchange. For this reason potassium uptake was measured by means of K^{42}, and is thus directly comparable with that of Cs^{137}. This method also provides a sensitive measure of the potassium absorbed from low concentrations, which may be small in relation to the original content of the plant. It is convenient then to express the relative rates of uptake of Cs^{137} and K^{42} in terms of the observed ratio (O.R.) given as

$$\frac{Cs^{137}/K^{42} \text{ plant}}{Cs^{137}/K^{42} \text{ solution}}.$$

The O.R. has widely been used in comparisons of the behavior of radioactive strontium and calcium (5). Values of less than unity indicate a more rapid rate of uptake of potassium.

MATERIALS AND METHODS

Excised roots of Tennessee Winter barley, 1956 crop, were prepared by the method of Jacobson et al (8). The method of measuring absorption is described in detail in a publication in preparation by Handley and Overstreet. Briefly, 1 g of root was immersed in 1 liter of solution in a polyethylene vessel. The solutions were aerated and held at 26° C. The initial pH was 5.5. After 1 hour the roots were removed, rinsed briefly, and prepared for assay.

The general technique of Hoagland and Broyer (7) was used to prepare whole barley plants. Barley seed (var. Arivat) was germinated on cheesecloth supported over distilled water by a metal screen. After 1 week the plants were transferred to Hoagland solution, one-eighth the standard concentration plus the necessary micronutrients, and were allowed to grow in the greenhouse for a further 12 to 14 days. The day before the experiments the plants were brought into the laboratory and left overnight under a bank of fluorescent lamps in a solution containing 1 meg/liter calcium chloride. It was thought that this procedure would enhance subsequent absorption.

The following morning, replicate groups of seven plants were transferred to plastic beakers containing 200 ml of solution of the same composition as the experimental solutions but with no added radioactivity. The plants were allowed to adjust to these conditions for 1 hour and were then transferred to radioactive solutions. Three replicates of seven plants each were used for each treatment. In most cases the experiment was ended after 2 hours in the radioactive solutions. Where low concentrations were used, the

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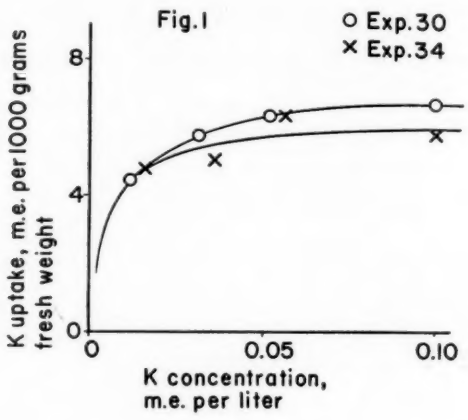
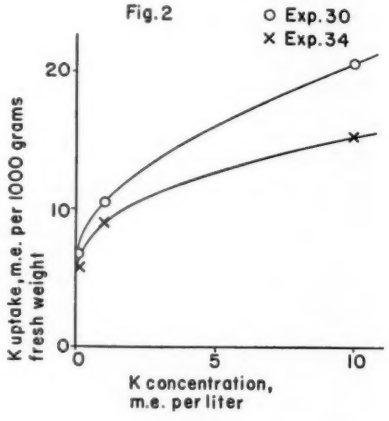
solutions were renewed once or twice in an attempt to prevent severe depletion. The plants were illuminated with fluorescent light throughout and were at room temperature. Unless otherwise stated, experimental solutions contained only potassium chloride in varying concentrations, calcium chloride (1 meq/liter), Cs^{137}, and K^{42}. Cs^{137} was obtained in carrier-free form, as CsCl in HCl from Oak Ridge National Laboratory. K^{42} was obtained as a solution of potassium chloride in HCl, the specific activity of which was about 25 mc/g when used. Cs^{137} was used at a concentration of 10 μc/liter, K^{42} at 10 to 20 μc/liter.

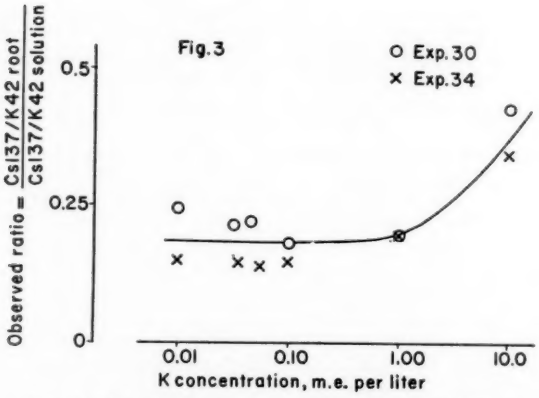
For assay, samples of initial and final solutions were evaporated on small nylon planchets. Roots and shoots were dried and packed into the same type of planchet and covered with mylar film. Assays of γ-activity were made by a scintillation counter. Counting immediately gave a measure of the combined count-rate of K^{42} and Cs^{137}. A second assay after 5 days when the K^{42} (half life 12.44 hr) had decayed to an insignificant amount gave an estimate of the count-rate of Cs^{137} (half life 30 yr). From these two measurements, with appropriate corrections for decay, the relative count-rates of K^{42} and Cs^{137} in the plants and in the solutions were calculated. To calculate the O.R., the ratio of Cs^{137} to K^{42} in the solutions was based on mean concentrations during the experimental period. Total uptakes of stable potassium and cesium were calculated by direct proportion from the count-rate of K^{42} and Cs^{137}. For the intact plants potassium and cesium uptakes are expressed as microequivalents per seven plants; corrections for differences in weight were not made.

Results

The effect of the external concentration of potassium on the uptake of potassium, as measured by K^{42}







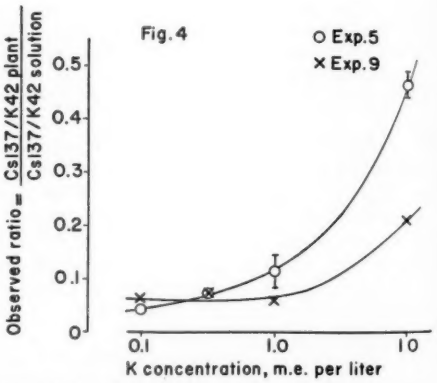


Fig. 1. The absorption of potassium by excised barley roots from solutions of KCl up to 0.1 meq/l.
Fig. 2. The absorption of potassium by excised barley roots from solutions of KCl up to 10 meq/l.
Fig. 3. The observed ratio of Cs^{137} to K^{42} in excised barley roots in concentrations of KCl up to 10 meq/l.
Fig. 4. The observed ratio for the transfer of Cs^{137} and K^{42} to the shoot of young barley plants in solutions of KCl up to 10 meq/l.

TABLE I

RELATIVE UPTAKE OF Cs^{137} AND K^{42} BY ROOTS OF INTACT BARLEY PLANTS

EXPT.	EXTERNAL K CONC= 10 MEQ/L		EXTERNAL K CONC= 0.1 MEQ/L	
	K UPTAKE***	O.R.	K UPTAKE***	O.R.
4	29*	0.31	13	0.11
	51**	0.31	16	0.24
5	37	0.22	20	0.29
6	23	0.34	12	0.34
7	31	0.20	19	0.26
8	57	0.16	26	0.18
9	70	0.13	40	0.25
Mean		0.24		0.24

* Grown in full nutrient solution.
** Grown in nutrient solution low in potassium.
*** Microequivalents/7 plants.

and on the O.R. in excised roots is shown in figures 1 to 3. If low concentrations alone are considered, it appears that potassium uptake rose to an apparent maximum at about 0.1 meq/liter (fig 1). However, it is clear from figure 2, that as the concentration was further increased, potassium uptake by the roots also increased and had not reached a maximum at a concentration of 10 meq/liter. The O.R. for excised roots did not consistently differ over the range 0.01 to 1.0 meq/liter (fig 3); above this concentration, however, the selectivity for potassium decreased, the O.R. rising to about 0.4.

Tables I and II summarize the results of seven experiments with intact barley plants in all of which external concentrations of potassium of 10 meq/liter and 0.1 meq/liter were used. For the roots the mean O.R. was 0.24 irrespective of concentration, but for the shoots, the external potassium concentration had a most marked effect on the O.R. At 10 meq/liter the selectivity for K^{42} in transfer to the shoots was low (mean O.R. = 0.5), but at a concentration of 0.1 meq/liter K^{42} was apparently transported at 10 to 20 times the rate of Cs^{137}. In two experiments the O.R. of the shoot was measured over a range of concentrations. The results are shown in figure 4. Differences in the O.R. up to 1.0 meq/liter are not statistically significant; however, the O.R. is significantly higher at 10 meq/liter. Attempts to measure the O.R. at lower concentrations than 0.1 meq/liter were unsuccessful because of marked depletion of the solution.

The O.R. was also measured for solutions containing K^{42} and Cs^{137}, with both stable potassium and cesium added in the following ratios: 1:0, 0:1, or 1:1, at total concentrations of 10, 1, or 0.1 meq/liter. Because the solution of K^{42} also contained some stable potassium, the actual ratios of potassium to cesium were those given in tables III and IV. In excised roots, substituting stable cesium for potassium led to an increased percentage uptake of both K^{42} and Cs^{137}, though proportionately more for the former. This led to a decrease in the O.R. Where an equal mixture of cesium and potassium was used, the results were intermediate. Generally similar results are obtained with the roots of intact plants, but at the lower concentration the O.R. rose in the presence of cesium carrier. In this case, changing the solution was not effective in preventing severe depletion of the K^{42}, this probably considerably reduced the rate of uptake of K^{42} and this increased the O.R.

In transfer to the shoots, a similar decrease in the O.R. in the presence of cesium was shown only at the high concentrations; at neither concentration, however, did the differences reach statistical significance. The O.R. appeared to be determined more by the total external concentration than by the relative amounts of potassium and cesium present in the culture solution.

In two early experiments, O.R.'s greater than one were observed in the shoots (table V). Comparison of the potassium uptake with the values in tables I and II shows that these plants absorbed comparatively slowly. Lack of exudation from the cut shoots and poor lighting conditions in the experiment suggested that the high O.R. observed was a result of a slow rate of water movement. Two later experiments examined the effect of shading and of mannitol in the external solution on the O.R. (table VI). Shading, which reduced water movement by approximately 70 per cent, did not greatly affect uptake or the O.R.; mannitol on the other hand had a severe effect on water uptake and greatly reduced transport to the shoot. The O.R. increased markedly, rising above one in the higher concentration. Potassium uptake by the roots was reduced in the presence of mannitol, but the effect on the O.R. was not large.

TABLE II

RELATIVE TRANSPORT OF Cs^{137} AND K^{42} TO SHOOTS OF YOUNG BARLEY PLANTS

EXPT.	EXTERNAL K CONC= 10 MEQ/L		EXTERNAL K CONC= 0.1 MEQ/L	
	K UPTAKE***	O.R.	K UPTAKE***	O.R.
4	7.2*	0.52	1.0	0.061
	17.0**	0.29	4.6	0.057
5	6.5	0.47	1.1	0.043
6	3.0	0.63	0.83	0.074
7	2.0	0.57	0.67	0.061
8	2.9	0.82	0.32	0.091
9	2.2	0.21	1.6	0.061
Mean		0.50		0.064

* Grown in full nutrient solution.
** Grown in nutrient solution low in potassium.
*** Microequivalents/7 plants.

TABLE III

VARYING RATIOS OF POTASSIUM AND CESIUM IN EXTERNAL SOLUTION ON UPTAKE OF K^{42} AND Cs^{137} BY EXCISED ROOTS

TOTAL CONC = 10 MEQ/L				TOTAL CONC = 0.1 MEQ/L			
K/Cs RATIO	% UPTAKE		O.R.	K/Cs RATIO	% UPTAKE		O.R.
	K^{42}	Cs^{137}			K^{42}	Cs^{137}	
1:0	1.1	0.20	0.17	1:0	7.8	1.5	0.19
1:500	6.4	0.69	0.11	1:50	32.	4.4	0.14
1:1	1.7	0.23	0.13	1:1	12.	2.0	0.16

DISCUSSION

In the experiments described above, except under limited circumstances, barley plants absorbed K^{42} to a greater extent than Cs^{137}, relative to the concentration in the external solution. A very wide variation in the ratio of stable potassium and cesium at any given concentration had a relatively small effect on the observed ratio of Cs^{137} to K^{42}. From this it may be concluded that absorption of carrier-free Cs^{137} in the presence of potassium does not differ in kind from that of macro amounts of cesium and that the results of experiments using carrier-free levels may be of general qualitative applicability. Stable potassium competed with K^{42} and Cs^{137} for uptake to a greater extent than did cesium. Thus a greater percentage of both K^{42} and Cs^{137} was absorbed by roots from a solution of cesium than of potassium. However, the lower O.R. in cesium indicated that the cesium competed more effectively with Cs^{137} than with K^{42}.

The concentration of the external solution affected the O.R. in excised roots to some extent. Selectivity appeared to be greater over the range 0.01 to 1.0 meq/liter than at 10 meq/liters (fig 3). It was also observed that, in excised roots, potassium uptake, as measured by K^{42}, appeared to level off at a comparatively low level of concentration and then to show a further rise as the concentration was again increased. This is similar to the behavior observed for cesium by Bange and Overstreet (2) and tentatively ascribed by them to the involvement of two processes in uptake, one saturated at relatively low levels, the other important only as the concentration rises. If this postulate is accepted, it appears that the two processes differ also in their selectivity for potassium and cesium.

The O.R. in the roots of intact plants appeared to be independent of the concentration of potassium (table I) and approximated that found for excised roots in the low range of potassium concentration. One might speculate that transfer to the shoot results in a more efficient operation of the postulated first mechanism for potassium absorption by entailing a more rapid release of potassium after absorption. If this is the case, competition for the first mechanism would be observed at higher concentrations of potassium. Alternatively, it is conceivable that substrate supplied by the shoot enhances the activity of the first mechanism. However, the reason for this difference in behavior between excised and attached roots remains obscure.

A similar influence was demonstrated in a more marked form in transfer from root to shoot (table II). The degree of selectivity for K^{42} and Cs^{137} appeared to be highly dependent on the total external concentration, whether of the two elements singly or in combination, rather than on their actual proportions. This renders untenable explanations based on a less effective dilution of Cs^{137} than of K^{42} as the concentration of potassium increases; a similar increase in O.R. was found when cesium was increased. As with the excised roots, however, the observations

TABLE IV

VARYING RATIOS OF POTASSIUM AND CESIUM IN SOLUTION ON UPTAKE OF K^{42} AND Cs^{137} BY ROOTS AND SHOOTS OF YOUNG BARLEY PLANTS

TOTAL CONC = 10 MEQ/L				TOTAL CONC = 0.1 MEQ/L			
K/Cs RATIO	% UPTAKE		O.R.	K/Cs RATIO	% UPTAKE		O.R.
	K^{42}	Cs^{137}			K^{42}	Cs^{137}	
Shoot							
1:0	0.14	0.11	0.82 ± 0.18	1:0	1.4	0.25	0.091 ± 0.022
1:1000	0.16	0.075	0.49 ± 0.12	1:10	2.7	0.36	0.093 ± 0.011
1:1	0.22	0.12	0.59 ± 0.04	1:1	3.2	0.38	0.069 ± 0.016
Root							
1:0	2.8	0.39	0.16 ± 0.03	1:0	64	24	0.18 ± 0.02
1:1000	11.8	1.27	0.11 ± 0.01	1:10	96	36	0.28 ± 0.02
1:1	3.9	0.50	0.15 ± 0.05	1:1	97	40	0.22 ± 0.02

TABLE V

POTASSIUM UPTAKE AND OBSERVED RATIO OF Cs^{137} AND K^{42} IN SLOWLY ABSORBING PLANTS

EXPT	ABSORPTION PERIOD, HR	EXTERNAL K CONC = MEQ/L		EXTERNAL K CONC = 0.1 MEQ/L	
		K UPTAKE*	O.R.	K UPTAKE*	O.R.
2 (shoots)	1	0.13	2.4	0.0047	1.7
3 (shoots)	2	0.52	1.7	0.034	0.41
3 (roots)	2	4.5	0.85	0.76	0.33

* Microequivalents/7 plants.

are compatible with the existence of two processes of uptake. The process predominating at low concentrations is very highly selective for potassium; that at higher concentrations is much less selective.

The detailed experimental evidence on which to base such a hypothesis is not yet available. An obvious implication of these results is, however, that if the selectivity of plants for alkali cations is to be studied, a wide range of concentrations should be used. Certainly experimentation should not be confined to concentrations above 1 meq/liter; even the concentrations of normal nutrient solutions may be too great for full selectivity to be shown.

It is interesting at this point to compare these observations with those of previous workers obtained by growing a number of species of plants over an extended period. Collander (4) used a nutrient solution containing 2 meq/liter of potassium; an average O.R. for many species of 0.8 (0.5–1.3 range) can be calculated from his data. Menzel and Heald (9) with a potassium concentration in the solution of 2.8 meq/liter found the O.R. to vary from 0.1 to 0.3. These latter values are comparable with those obtained at high concentrations in our experiments at an equivalent concentration of potassium; those of Collander are rather greater, but a high order of accuracy was not claimed for these values. Some recent results of Cline and Hungate (3) have kindly been made available to us. These authors grew bean plants for 16 days in a nutrient solution, the potassium concentration of which was varied over a wide range. Cs^{137} was added and the O.R. of Cs^{137} to stable potassium determined. The experimental treatments led to differences in growth rates but in spite of this and the differences in technique, the results of their experiments are in remarkable agreement with the data given above. This encourages the view that the results are of general applicability.

The slow rate of water movement and the known preference of the cells for potassium explain the high values of the O.R. observed in plants in which uptake is slow or in which the water uptake is inhibited by mannitol. Conditions of very slow transport either across the root or through the xylem, may have provided opportunities for a relatively large proportion of the ions to be absorbed by the cells of the root. With continued selection in favor of K^{42}, the solution reaching the shoot would thus have been relatively enriched in Cs^{137}. Under conditions of rapid transport the opportunity for absorption by intervening cells would have been reduced. Against this simple explanation is the fact that shading reduced water movement but did not affect uptake or the O.R. Further, there is no direct evidence that the high O.R. in the early experiments was due to a low rate of water movement rather than a low rate of uptake per se. It is possible that high selectivity for potassium is correlated with the phenomenon of root pressure, which may be manifested by a high rate of uptake and which is inhibited by the presence of mannitol in the solution.

TABLE VI

MANNITOL AND SHADING EFFECT ON POTASSIUM UPTAKE AND OBSERVED RATIO OF K^{42} AND Cs^{137} IN YOUNG BARLEY PLANTS

EXPT.	TREATMENT	K CONC = 10 MEQ/L		K CONC = 0.1 MEQ/L	
		K UPTAKE*	O.R.	K UPTAKE*	O.R.
6 (shoot)	None	3.0	0.63 ± 0.05	0.83	0.074 ± 0.004
	Mannitol (0.25 M)	0.25	1.9 ± 0.30	0.016	0.75 ± 0.17
6 (root)	None	23	0.34 ± 0.06	12	0.34 ± 0.03
	Mannitol (0.25 M)	17	0.48 ± 0.06	6.3	0.30 ± 0.005
7 (shoot)	None	2.1	0.57 ± 0.01	0.67	0.061 ± 0.006
	Shading	2.9	0.42 ± 0.11	0.71	0.041 ± 0.009
	Mannitol (0.25 M)	0.3	1.25 ± 0.00	0.005	0.90 ± 0.18
7 (root)	None	31	0.196 ± 0.013	19	0.156 ± 0.017
	Shading	44	0.294 ± 0.008	21	0.151 ± 0.015
	Mannitol (0.25 M)	29	0.203 ± 0.045	10	0.232 ± 0.023

* Microequivalents/7 plants.

Summary

The relative rates of uptake of potassium and cesium by excised roots and by intact plants of barley from solutions have been measured by K^{42} and Cs^{137}. The results have been expressed as the observed ratio (O.R.):

$$\text{O.R.} = \frac{Cs^{137}/K^{42} \text{ plant}}{Cs^{137}/K^{42} \text{ solution}}$$

In excised roots, in external concentrations of potassium up to 1.0 meq/liter, the O.R. was in the range of 0.15 to 0.25. At a higher concentration (10 meq/liter) the O.R. was approximately 0.4, thus indicating a lower selectivity for potassium at this level. In the transfer from root to shoot a marked effect of potassium concentration on the O.R. was observed. At a concentration of 0.1 meq/liter, K^{42} was transferred at 10 to 20 times the rate of Cs^{137} (mean O.R. = 0.06). At 10 meq/liter, K^{42} was transferred at only twice the rate of Cs^{137} (O.R. = 0.5). When the proportions of added stable potassium and cesium in the medium were varied over a very wide range, the O.R. was affected by a factor of less than two. When the rate of potassium uptake was low or when water movement was restricted, the O.R. in the shoots was greater than unity, showing a greater transference of Cs^{137}.

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GROSS NUTRITIONAL CONTRIBUTIONS OF MAIZE ENDOSPERM AND SCUTELLUM TO GERMINATION GROWTH OF MAIZE AXIS [1, 2]

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In 1890 Brown and Morris (8) showed that starch paste, when applied to the absorbing surface of the scutellum, could replace the endosperm during the germination of barley embryos. Andronescu (2) first demonstrated that the maize embryo (axis & scutellum) could develop into a normal plant when separated from its endosperm and supplied with sucrose. Most investigators, however, have emphasized the endosperm as a source of growth factors, either essential or stimulatory to the germination of the axis, rather than as a tissue serving singularly as a source of sugars and inorganic ions. Schander (26) considered the aleurone layer of the maize endosperm to be the source of an accelerator which is absorbed by the scutellum during germination. DeRopp (11) found that embryos which were allowed to remain in the intact grain during the early stages of germination exhibited more vigorous growth after excision than did embryos removed from the dry rye seed. He also interpreted this as due to the absorption by the scutellum early in germination of a growth factor from the endosperm. The "Z" factor of Robbins (25) and the "blastinin" hormone of Cholodný (10) are other compounds said to originate in the endosperm. The high percentage of bound auxin residual in the resting endosperm of maize (3, 4, 7, 13, 14), and the increasing discoveries of gibberellin-like substances in the seeds of higher plants (22, 24) also have made the contribution by the endosperm to the growth of the axis seemingly more complex than merely that of a sugar and inorganic ion source. Furthermore, Nason (19) found that the rate and extent of niacin accumulation in intact maize embryos were three times as great as that of excised embryos after 10 days of germination. His data suggest that this is related to the absorption of tryptophan from the endosperm rather than to the absorption of niacin itself.

The extent to which the scutellum can provide nutrition to the axis has received little attention, although it has been shown that lipids are rapidly lost from the maize scutellum during germination (18).

The object of this research was to determine the relative nutritional contributions of the scutellum and the endosperm of the maize kernel to the growth of the axis during germination.

METHODS AND MATERIALS

The material used was a non-waxy, inbred maize (*Zea mays* L.) line T854, secured from the Agricultural and Mechanical College of Texas. The endosperm of this line contain 85 % carbohydrate and 10 % protein on a dry weight basis (12). Oil is present in trace amounts in the aleurone layer, demonstratable by staining with Sudan IV. The predominate carbohydrates found in non-waxy maize endosperm during germination are starch, maltose, dextrins of various chain lengths, and a trace of glucose (29, 6). Amylose comprises about 25 % of the starch of non-waxy maize endosperm and the remainder is amylopectin (20, 21). The scutellum of the T854 line contain 27 % lipid,, 22 % protein, and 35 % carbohydrate on a dry weight basis (12). Sucrose is the principle sugar in the scutellum of germinating maize, and there is no detectable maltose in this organ (29).

The contributions of the endosperm and scutellum to axis growth were determined by comparing the axis growth of intact seeds with that of excised embryos and excised axes cultured on synthetic media. The germination period of this line of maize was arbitrarily defined as the 12 days following the initial imbibition of water by the seed. The stored nutritional reserves of this line will support a logarithmic rate of growth by the axis (as determined by dry wt) for this period in darkness at the temperature used. After 12 days this growth in darkness tapers off considerably. The initial imbibition of water by the seed which marks the commencement of germination was arbitrarily considered to begin as soon as the seeds were wetted.

The seeds were culled to a standard type kernel appearance and size. Those used to determine the growth rate of the axis in the normal germination of intact kernels (hereafter referred to as the intact-kernel culture) were disinfected by washing with "Tide" and rinsing with distilled water. These seeds were then germinated on agar in 25 ml micro-Fernback vessels, since these vessels were used in the in vitro cultures. In the cultures of excised axes the

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dissection effected a clean separation from the scutellum. In the cultures of entire embryos, the adhering endosperm was gently scraped away. In both cases the kernels were presoaked 1 hour, this in order to loosen the pericarp; neither the embryo nor the endosperm showed appreciable water imbibition. It was considered that there was no exchange of materials between the scutellum and the endosperm before excision. Normal aseptic procedures were employed. All cultures were grown at 21° C in total darkness.

The salt content of the medium was a modification of that of Randolph and Cox (23) (table I). In no case was the medium supplemented with amino acids, vitamins or other factors, but rather, deliberately maintained as minimal as possible. The medium was jelled with 0.75 % acid-washed Noble Agar and 10 ml added to each vessel. The pH of this medium was 5.0 regardless of the carbohydrate substrate used and was relatively stable throughout the germination period.

The following cultures were effected:

Culture designation	Composition of medium
Intact-kernel culture (entire kernels)	...
Axis culture (excised axis)	Salt solution, 2 % sucrose
Non-nutrient culture (entire embryo)	Agar only
2 % Maltose culture (entire embryo)	Salt solution, 2 % maltose agar
2 % Sucrose culture (entire embryo)	Salt solution, 2 % sucrose agar
5 % Maltose culture (entire embryo)	Salt solution, 5 % maltose agar
2 % Glucose culture (entire embryo)	Salt solution, 2 % glucose agar
2 % Soluble starch culture (entire embryo)	Salt solution, 2 % soluble starch, agar
Starch paste culture (entire embryo)	Salt solution on filter paper, soluble starch on scutellum

Table I

Salt Content of Media Employed in All Cultures Except Intact-Kernel and Non-Nutrient Cultures*

	mg/liter
$Ca(NO_3)_2 \cdot 4H_2O$	237.0
KNO_3	85.0
KCl	65.0
$NaH_2PO_4 \cdot H_2O$**	16.5
$MgSO_4 \cdot 7H_2O$	36.0
Fe citrate (chelate)	30.0
$MnSO_4 \cdot 4H_2O$	00.4

* Modified from Randolph and Cox (23).
** Substitution of $NaH_2PO_4 \cdot H_2O$ for "Calgon".

The choice of substrates for the cultures was based on the fact that A, the bulk of the endosperm is starch (17, 29); B, the degradation product of the amylase digestion of the endosperm starch is primarily maltose (5, 15, 20); C, the only sugars found in gross amounts in the embryo, and, thus, considered in transport form, are sucrose and glucose (17, 29). The starch paste culture was devised to simulate physically the intact-kernel endosperm with respect to endosperm starch. Ten discs of Whatman No. 1 filter paper were placed in circular vegetable dishes and saturated with 20 ml of the salt solution. Small glass microscope slide rings were packed with soluble starch paste and placed on the filter paper. The embryos were embedded in this simulated endosperm. The flat, external surface of the scutellum from which the axis emerges was exposed, allowing the shoot and root to grow out of the ring. This procedure eliminated the artificialities of root sugar absorption and of the substrate becoming limiting in the area of the absorbing surface of the scutellum.

The germination growth accomplished in the different cultures was determined by measuring the increase in fresh and dry weight of the axes. In each culture, 30 embryos or axes were used and samples removed for weight determinations at 3, 5, 8, 10, and 12 days after germination began. All experiments were repeated, and the results of the replications were within 10 % of the original culture. The data are averages of the two runs of each culture. Since the dry weight percentage of fresh weight for the axes was no different in any of the cultures, only axis dry weight values are given in the results.

Fresh and dry weight changes in the scutellum were followed at the same intervals during germination in the intact-kernel culture and the other cultures which employed the entire embryo. Total nitrogen content of the scutellum was determined for the intact-kernel culture, the non-nutrient culture, and the 5 % maltose culture, and of the endosperm from the intact-kernel culture at these same intervals. Nitrogen was determined by the modified micro-Kjeldahl method of Jensen (16).

Lipid content of intact-kernel scutella was measured at 24-hour intervals during the first 6 days of germination. The scutella were sliced, oven dried, and the lipids extracted by repeated washings with an ether: ethanol (3:1) solvent at 45° C.

The respiratory quotient and total respiration of embryos dissected from intact kernels were also determined for the 12-day germination period, utilizing standard Warburg manometry. Gas exchange was followed for 2 hours in embryos removed from intact seeds germinating in the dark. The embryos were placed on a film of water in the Warburg vessels, but were not submerged. The uptake of O_2 was measured directly by absorbing the CO_2 evolved with 15 % KOH. CO_2 evolution was measured by the "direct method" as described by Umbreit et al (30).

Results

1. Comparison of Axis Growth Obtained in Intact-Kernel Culture With That in in Vitro Cultures (figs 1–5). The results of the intact-kernel culture (fig 1, curve 1) establish the growth pattern of the germinating axis in the intact seed and serve as a control to which the growth in other cultures is compared. Figure 1, curve 2, reveals the growth pattern of the non-nutrient culture. The growth of the axis supported by its scutellum alone equals the growth of the intact-kernel culture for the first 5 days. But after the 5th day this growth diminishes rapidly, and by the 12th day these axes have only 22 % of the intact-kernel culture dry weight. The results of the axis culture, (fig 1, curve 3) reveal that the maize axis without its scutellum is capable of very little growth on a sucrose and salt medium. After 12 days the dry weight of these axes is less than 10 % of that of the intact-kernel culture and only 41 % of that of the non-nutrient culture.

However, when samples of the non-nutrient culture (axis & scutellum germinated on 0.75 % agar alone) are cultured in the light they develop into autonomous plants. Thus, the embryo with no nutrient other than what it contains in its scutellum can germinate successfully. Samples of the axis culture (dissected axis germinated on the salt and 2 % sucrose medium) will also develop into autonomous plants when cultured in the light. These excised axes in the light grow slowly, but their growth rate increases greatly when photosynthesis begins.

The 2 % maltose and the 2 % sucrose cultures can be discussed collectively since their growth rates are essentially identical (fig 2). These cultures reveal that sucrose, which is not found at maturity, nor throughout germination, in the endosperm (6, 17, 29), can be absorbed by the scutellum as readily as maltose. The data show that the axis growth on 2 % disaccharide media is more than 175 % of the intact-kernel culture growth by the 5th day. However, these media provide for only 75 % of the intact-kernel growth by the 12th day. This enhanced early growth is probably due to the ready availability of sugar in these cultures during the first few days of germination. A decreasing availability of sugar with time in the area of the scutellar absorbing surface is considered responsible for the tapering off of growth in these two cultures during the last phases of germination.

The axis growth in the 5 % maltose culture (fig 3) equals the growth found in the intact-kernel culture during the first 8 days, but it declines to 84 % by the 12th day. The axes of this culture in comparison to those of the 2 % disaccharide cultures show a lower initial growth rate, which could conceivably be due to the higher osmotic pressure of the medium. They exhibit less of a tapering off of growth during the last phases of germination, which may be explained by a greater availability of sugar at this stage than is afforded by a 2 % concentration.

The results of the 2 % glucose culture (fig 4) demonstrate that this sugar provides the most satisfactory sugar substrate of those used, since growth in this culture equals the intact-kernel growth after 12 days. A comparison of this culture with the intact-kernel growth after 5 days shows again that greater early growth can be correlated with sugar being readily available for absorption by the scutellum during the first days of germination.

The 2 % soluble starch culture (fig 5 curve 3) gives only 50 % of intact-kernel growth after 12 days. This poor growth may result from a poor diffusion of starch and, hence, an increasing sugar gradient at the scutellar surface. The starch paste culture (fig 5 curve 2) essentially follows the intact-kernel growth pattern throughout the germination period, including the early lag phase, when compared to embryos cultured on sugars.

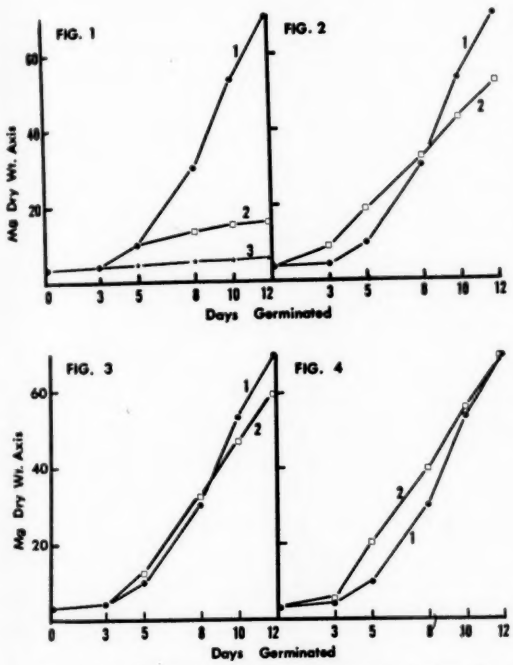

Fig. 1. Axis growth (dry wt increase) during germination. Curve 1, intact-kernel culture (control). Curve 2, non-nutrient culture. Curve 3, axis culture.

Fig. 2 Axis growth (dry wt increase) during germination. Curve 1, intact-kernel culture (control). Curve 2, average of 2 % maltose and 2 % sucrose cultures.

Fig. 3. Axis growth (dry wt increase) during germination. Curve 1, intact-kernel culture (control). Curve 2, 5 % maltose culture.

Fig. 4. Axis growth (dry wt increase) during germination. Curve 1, intact-kernel culture (control). Curve 2, 2 % glucose culture. (See text for details of culture conditions.)

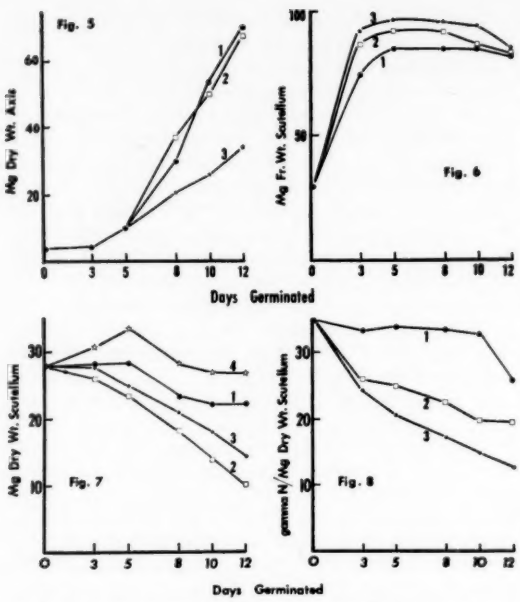

FIG. 5. Axis growth (dry wt increase) during germination. Curve 1, intact-kernel culture (control). Curve 2, starch paste culture. Curve 3, 2 % soluble starch culture.

FIG. 6. Fresh weight of the scutellum during germination. Curve 1, intact-kernel culture (control). Curve 2, average of non-nutrient, 2 % sucrose, 2 % maltose, 2 % soluble starch cultures. Curve 3, Average of 5 % maltose, 2 % glucose, starch paste cultures.

FIG. 7. Dry weight of scutellum during germination. Curve 1, intact-kernel culture (control). Curve 2, non-nutrient culture. Curve 3, average of 2 % maltose, 2 % sucrose, 2 % soluble starch cultures. Curve 4, average of 5 % maltose, 2 % glucose, starch paste cultures.

FIG. 8. Scutellum nitrogen during germination. Gamma of nitrogen per mg. dry weight. Curve 1, intact-kernel culture (control). Curve 2, non-nutrient culture. Curve 3, 5 % maltose culture. (See text for details of culture conditions.)

II. SCUTELLUM WEIGHT CHANGES (figs 6, 7). There is a constant rise in the fresh weight of the scutella of intact kernels up to the 5th day, and the weight then reached is maintained throughout the experimental period (fig 6). The dry weight of the intact-kernel scutellum remains constant for the first 5 days of germination and then decreases gradually (fig 7).

The results of the other cultures fall into three groups. In the non-nutrient culture the scutellar fresh weight approximates that of the intact-kernel culture, but its dry weight decreases much faster. Thus, if no other source of nutrition is available, the demands of the axis deplete the scutellum to a greater degree. In the 2 % disaccharide and soluble starch cultures, the scutellar fresh weights also approximate those of the intact-kernel culture, and the scutella lose more dry weight than do the intact-kernel scutella, although not to the extent that those grown on the non-nutrient culture do. Hence, on these substrates, at this concentration, the scutellum is depleted somewhat more than it is in the intact seed. In the 5 % maltose, the 2 % glucose, and the starch paste cultures, all of which approximate the intact-kernel growth of the axis, both scutellar fresh weight and dry weight exceed that of intact-kernel scutella. Apparently, these substrates at these concentrations are absorbed faster than they are transported to the axis and thereby increase the scutellar dry weight, and, at the same time, increase the osmotic pressure of the scutellum, which results in a greater water holding capacity and a higher fresh weight.

III. SCUTELLUM AND ENDOSPERM NITROGEN (figs 8–10). Total nitrogen content of the scutellum was determined for the intact-kernel, non-nutrient, and 5 % maltose cultures. In figure 8, nitrogen is expressed as micrograms per milligram dry weight. The data reveal that during germination in the non-nutrient and the 5 % maltose cultures nitrogen is lost from the scutellum faster than dry weight is lost. It is presumed that this loss represents a transfer to the axis. The fact that nitrogen per milligram dry weight is lost faster in the 5 % maltose culture than in the non-nutrient culture is deceiving, since the non-nutrient culture is not replenishing its dry weight by absorption from the medium, and therefore its dry weight is much lower than is that of the 5 % maltose culture. Nevertheless, the data show that much more nitrogen is absorbed from the endosperm than is available in the medium, since scutella from the 5 % maltose culture are depleted of nitrogen to a greater extent than are the intact-kernel scutella. A better evaluation of the loss of nitrogen from the scutellum during germination is obtained by plotting micrograms of nitrogen per scutellum (fig 9). This plot shows that by the 12th day the scutella cultured in the non-nutrient medium have lost 79 % of their nitrogen, those cultured on 5 % maltose have lost 59 %, while the intact-kernel scutella have lost but 27 %.

In figure 10, micrograms of nitrogen in the endosperm of the intact-kernel culture are plotted per milligram dry weight. After an initial lag period, there is a steady loss of nitrogen from the endosperm from the 3rd to the 10th day of germination. By the 10th day the endosperm has lost 71 % of its nitrogen per milligram dry weight, but loses little more (73 %) after the next 2 days. No logical explanation for the initial rise in nitrogen content can be given.

IV. LIPID AND RESPIRATION RESULTS (fig 11, table II). The results of the lipid extractions from intact-kernel scutella are presented in Table II, and are in agreement with similar work on maize by Malhotra (18). The data show that the lipid content of the scutellum decreases from 27 % to 12 % (dry wt basis), during the first 3 days of germination. During the next 3 days, the lipid content remains

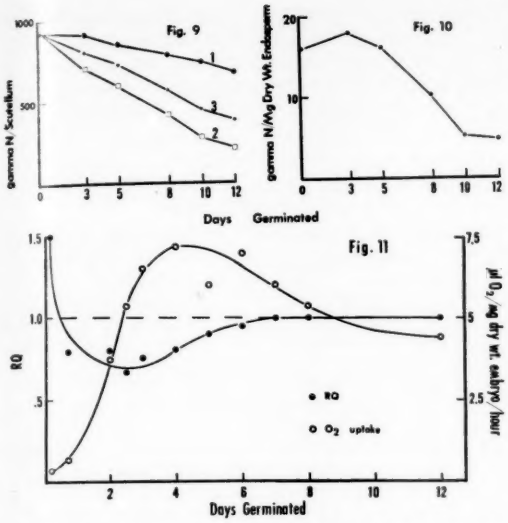

FIG. 9. Scutellum nitrogen content during germination. Gamma of nitrogen per scutellum. Curve 1, intact-kernel culture (control). Curve 2, non-nutrient culture. Curve 3, 5 % maltose culture. (See text for details of culture conditions.)

FIG. 10. Endosperm nitrogen content during germination. Gamma of nitrogen per mg dry weight.

FIG. 11. Respiratory quotient and oxygen uptake of intact-kernel embryos during germination. Dashed line is reference line denoting respiratory quotient of unity.

constant, indicating that lipids and material contributing to the dry weight of the scutellum are being lost at an equal rate.

The uptake of O_2 per milligram dry weight of the intact-kernel embryo over the 12-day germination period is given in figure 11. The total respiration of the embryo rises continually, but in relation to the increase in dry weight of the embryo a definite peak in respiration is reached on the 4th day. The decrease from the 4th to the 12th day may be caused by a gradual depletion of available carbohydrates. The respiratory quotient values for the intact-kernel embryo over the germination period are also presented in figure 11. The first respiratory quotient, that for 4 hours, shows that part of the embryo respiration at this early stage is still anaerobic, but by 18 hours the influence of lipid breakdown is such that the respiratory quotient is well below unity. The rise back to unity caused by the depletion of lipids and the increasing availability of carbohydrate is achieved by the 7th day at this temperature (21° C). This respiratory quotient pattern is in agreement with that of cereal germination in general (27).

DISCUSSION AND CONCLUSIONS

From this study concerning the nutritional contributions of the maize endosperm and scutellum to axis growth during germination, the following conclusions may be drawn.

First, the early germination growth of the axis (the first 5 days in this instance) apparently is dependent upon the scutellar food reserves. This conclusion is supported by the data in figure 1. The data show that the growth of the axis, when the embryo is cultured in the non-nutrient medium, is identical with the growth of intact-kernel axes for these first 5 days.

Second, this early growth of the axis is produced at the expense of the scutellar lipid reserves. This conclusion is supported by the data from figure 11 and table II. Table II shows that a rapid loss of lipid from the scutellum occurs during the first 3 days of germination, and the respiratory quotient values of the embryo (fig 11) are well below unity during this period, which suggests a predominately lipid catabolism. This view is further substantiated by the fact that there is very little amylase activity in the endosperm at 3 days and very little reducing sugar (12). It seems unlikely that a substantial amount of endosperm carbohydrate reaches the axis before the 5th day of germination at this temperature. Furthermore, the axes from the intact-kernel and the starch paste cultures show a definite growth lag during these first 5 days, when compared to axes from the 2 % maltose, 2 % sucrose, and 2 % glucose cultures. This growth lag may be caused by the time required for the scutellum to secrete amylases into the endosperm and absorb the sugars produced from the amylolysis of starch, which must occur in the intact-kernel and starch paste cultures, but not in the sugar cultures. It is during this period, in which the endosperm nutrients are becoming mobilized, that the lipid catabolism in the scutellum apparently provides the axis with the energy and synthesis substrates for its initial growth in the germination of intact seeds.

Third, the function of the endosperm during germination seems to be confined to that of a source of carbohydrate and inorganic ions. This conclusion is supported by the following facts, A, Growth in

TABLE II

LIPID CONTENT OF SCUTELLUM DURING GERMINATION*

UNGERMINATED	DAYS GERMINATED					
	1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	6 DAYS
27	16	14.5	12	11	11	11

* Expressed as percent of scutellar dry weight.

two cultures, 2% glucose and starch paste, both equalled the growth found in the intact-kernel culture, while growth in a third culture, 5% maltose, closely trailed it (figs 3, 4, & 5). The growth on lesser concentrations of disaccharides tended to lag only after the 8th day. B, the results of the axis culture and the non-nutrition culture demonstrate that the embryo without additional nutrition can grow into an independent plant; and the axis alone, if provided with sucrose and inorganic ions, can grow into a self-sustaining plant, if, in both cases, they are allowed to photosynthesize.

Unquestionably, many compounds other than sugars and inorganic ions are absorbed by the scutellum during germination (19), but their role as essential and non-synthesizable metabolites is questionable in light of these results.

Fourth, in these experiments the axis growth of excised embryos is essentially independent of the carbohydrate supplied. Nutrient media that contained 5% maltose, 2% glucose or starch paste in addition to inorganic salts were approximately equal to the endosperm of intact kernels in supporting axis growth (figs 3, 4, & 5). As shown by figure 2, axis growth of embryos cultured in media containing 2% maltose or 2% sucrose was exceeded by that of intact kernels only after the 8th day of germination.

Concerning the different substrates used in these cultures, the superiority of glucose for isolated cereal tissue has been shown by Burström (9), Almestrand (1), and others. Yet the growth sustained by glucose in this study is surprising for two reasons. A, only trace amounts of glucose are formed in the amylolysis of starch (15, 5), and this only from the occasional splitting of maltotriose into maltose and glucose by alpha amylase. B, if sugar absorption involves a phosphorylation at the cell surface (28, 31), a disaccharide (maltose in this case) should be a more efficient substrate than a monosaccharide such as glucose. The reaction of a hexoglucoside with inorganic phosphate requires little energy, whereas the phosphorylation of glucose alone is dependent upon ATP.

Sucrose is, in general, the best carbohydrate source for plant tissue culture (31), but the fact that it supports the same rate of growth as does an equal concentration of maltose in these experiments is also surprising. There is no trace of sucrose in the endosperm of germinating maize (6, 17).

Summary

The gross nutritional contributions to the germination growth of the maize axis by the scutellum and the endosperm were investigated by germinating excised embryos and excised axes on different carbohydrate substrates and following their growth. The results indicate that the mature embryo depends on the endosperm only for a source of carbohydrate and inorganic ions, and that it does not require hormones, vitamins, or other factors originating in the endosperm for successful germination.

Respiratory quotients for the embryo and the loss of lipid from the scutellum were determined at intervals during germination, and these data coupled with the results of the culture studies suggest that the early germination growth of the axis is dependent upon the lipid of the scutellum.

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SITE OF ORIGIN AND EXTENT OF ACTIVITY OF AMYLASES IN MAIZE GERMINATION[1,2]

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The means by which the endosperm starch of the cereal grain is mobilized and made available to the embryo during germination have received considerable attention. The enzymes involved in the degradation of starch and their specific functions have been extensively reviewed (1, 2, 4, 12, 18, 20, 24, 30). Of these enzymes, alpha and beta amylase are responsible for the bulk of the starch hydrolysis in the cereal endosperm. Other hydrolytic enzymes, Z, D, and R enzymes (1, 3, 24, 25), that cleave the 1-6 alpha glucosidic linkage and other rare and anomalous linkages, provide relatively small amounts of soluble carbohydrates.

The reports concerning the origin of the amylase enzymes within the tissues of the cereal grain itself present a somewhat confused and incomplete picture. Many investigators propose that the amylases originate, at least in part, in the endosperm itself, and ascribe to the endosperm the capability of autodigestion. Other workers conclude that no such capability exists in this tissue [reviewed in Miller (19)]. The finding of beta amylase activity in the subaleurone layers of the endosperm of resting wheat, barley, and rye grains (9) suggests the in situ origin of at least some amylolytic activity during germination.

Many workers consider the cells of the scutellar epithelium to be the source of the amylases, which are thought to be secreted by these cells into the endosperm during germination. The evidence for this secretion has been: A, light microscope observations of the behavior of the scutellar epithelium and of the apparent migration of particles from it into the endosperm during germination (13, 21, 28, 31, 32), B, the isolation of particles from the scutellum that contain amylolytic activity (11), C, the fact that excised cereal embryos will exhibit good germination growth on boiled endosperm and on starch paste (6, 8).

Bernstein (5) has suggested a separate origin for each of the amylases, based on genetic studies. He proposes that the inheritance of alpha amylase is an embryo characteristic (hence, secreted from the scutellum) and that the inheritance of beta amylase is a more maternal characteristic (formed in situ in the triploid endosperm). Most investigators agree on a scutellar origin of some or all of the amylolytic activity found in the cereal endosperm during ger-

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mination. Yet none of the proposals concerning the origin of these two enzymes are based on a quantitative comparison of the amylolytic activity secreted by the scutellum with the total amylolytic activity found in the endosperm throughout the germination period. Furthermore, there has been no attempt to separate the secreted activity or the in situ activity into that attributable to each of the amylase enzymes.

The objectives of this research are: A, to determine the total amylolytic activity in the maize endosperm during the germination period, B, to separate this activity into that attributable to alpha and beta amylase, and C, to determine the amount of alpha and beta amylase secreted from the scutellum and the amount arising in situ in the endosperm during germination.

Methods and Materials

The material used in this study was a non-waxy, inbred strain of maize (*Zea mays* L.) T854, secured from the Agricultural and Mechanical College of Texas. Germination methods and conditions have been previously outlined (8). For the amylase data to be reproducible at any point during the germination period, great care had to be taken in selecting the germinating seeds. The median fresh weight of the whole seed, of the scutellum, and of the axis had been previously determined for each interval during germination at which enzyme activity was to be assayed (8). Only when seedlings were used that demonstrated their similar physiological age in germination by having whole seed, scutellum, and axis fresh weights within 10 % of these median values could data be replicated. All the assays were performed three times and the results came within 15 % of the average of the three runs. The data presented in the results are the average of the three runs.

The following determinations of activity were performed:

I. The total amylolytic activity and the separate activities of alpha and beta amylase in the endosperm from intact seeds in the resting state and at 3, 5, 8, 10, and 12 days after germination commenced. (These data are hereafter referred to as those for "intact" endosperm.)

II. The total amylolytic activity and the separate activities of alpha and beta amylase from "germinating" endosperms from which the embryos had been removed before germination began at the same intervals during germination. (These data are hereafter referred to as those for "separate" endosperm.)

III. The total amylolytic activity and the separate activities of alpha and beta amylase secreted from dissected scutella into gelatin.

Enzyme Assay Procedure. Total amylolytic activity and the separate activities of alpha and beta amylase were assayed by a modification of the method described by Bernfeld (17). This procedure utilizes the reagent 3,5-dinitrosalicylic acid to measure the increase in reducing power of a soluble starch solution subsequent to incubation with the amylolytic enzyme(s). The brown reduction product was measured in a Bausch and Lomb Spectronic 20 at 540 mμ. With this method both alpha and beta amylase can be assayed separately and in combination, since their action in producing aldehyde endings is additive. The concentration of tissue was varied in order to maintain first order kinetics for the enzyme reaction. This was necessitated by the fact that the amount of activity per endosperm generally increased greatly as germination progressed. Therefore, at each interval in germination at which assays were to be made, it was determined what concentration of tissue substrate became limiting (an example of which is given in fig 1). The concentrations of tissue for the interval were kept well below this value thereafter. Special precautions are necessary to maintain first order kinetics in assaying the amylases, since the velocity of alpha amylase in cleaving the starch polymer decreases greatly as the average length of the polymer drops below ten glucose residues (12, 29). Also the velocity of beta amylase in cleaving maltose units from the polymer is thought to decrease as 1-6 alpha branch linkages are approached (1).

I. Total Amylolytic Activity. A. *Endosperm of intact seeds.* Three, four, or five endosperms (depending on the extent of activity at the different intervals during germination) were homogenized with unbuffered double distilled water in a Ten Broeck homogenizer submerged in an ice bath and brought to a final volume of 40 ml. The homogenate was centrifuged at 2,000 G for 10 minutes in a cold room. Since cereal amylases are soluble when active (20), and in preliminary work the spun-down pellet showed no activity, the pellet of debris was discarded. The supernatant was kept at 5° C in an ice bath and assayed within 1 hour after homogenation. The pH of this homogenate was 6.8 for the resting seed endosperm, and it dropped slightly with seedling age, reaching 5.9 in the 12-day preparations. Aliquots of 0.4 and 0.8 ml of the supernatant were removed, brought up to 1.0 ml with double distilled water, added to 0.5 ml of pH 5.0 citrate buffer (0.1 M), heated slowly to 30° C and mixed with 0.5 ml of 2 % soluble starch. The reaction mixture was maintained at 30° C for 3 minutes in a water bath at which time the reaction was stopped with 2 ml of the color reagent. The remainder of the assay strictly adhered to that outlined by Bernfeld. Neither calcium nor chloride ions were added to the reaction mixture, since cereal amylases (unlike amylases from other sources) have shown no requirement for these ions (4, 30).

The endogenous reducing sugars of the supernatant were determined by adding the color reagent to the 1.5 ml buffered aliquot (inactivating the enzymes) before adding the substrate. The standard was run with maltose and the enzyme activity expressed as mg maltose/minute/endosperm. Activity was not expressed per mg nitrogen since the nitrogen in the endosperm rapidly diminishes during germination (8).

B. *Endosperm separated from embryo.* These endosperms were assayed in the same fashion as were the endosperms from intact seeds.

C. *Scutellar secretion into gelatin.* Several methods were used in the attempt to capture the amylolytic activity secreted from the scutellum before a relatively successful one was devised. The secretion was initially demonstrated qualitatively by dissecting scutella from seeds that had been germinating 5 days and placing them in petri dishes containing 25 ml of 1 % agar and 2 % soluble starch. Twenty-four hours later the dishes were stained with an I_2KI solution (photo 1).

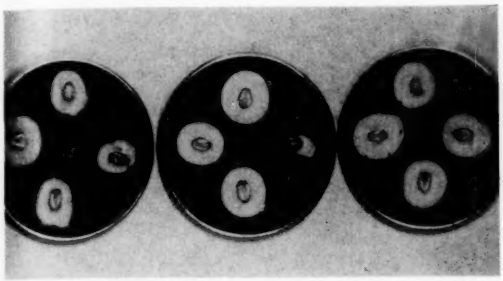


PHOTO 1. Demonstration of the secretion of amylase from the maize scutellum. Dishes contain gel made of 1 % agar, 2 % soluble starch into which scutella were embedded. Twenty-four hours later dishes were stained with I_2KI. Clear areas surrounding the scutella denote the absence of starch caused by amylase action.

In the initial attempt to measure quantitatively the amylase secreted, 30 scutella were dissected aseptically from dormant seeds and embedded in autoclaved vials containing 3 ml of 1 % washed agar. Samples were removed at intervals during the succeeding 12 days and the agar assayed for amylolytic activity. The results were in every case negative. This procedure was modified by leaving the axis attached to the scutellum and by adding starch to the agar (in the event the secreted amylase required contact with its substrate for activation, as is thought to be the case in *Pseudomonas saccharophila* (27). The results of these assays were still negative. Finally, scutella were removed from seed that had been germinating 3 days, placed on the agar, and then the agar assayed at intervals over the next 9 days. Amylolytic activity could then be found in the agar. Leaving the axis attached to the scutellum or incorporating starch into the agar had no effect on the extent of this activity. This requirement that the scutellum and the endosperm remain in contact during the initial stages of germination had been suggested by Brown and Morris (6), who postulated that the absorption by the scutellum of some nitrogenous compound(s) from the endosperm was necessary to initiate the secretion of amylolytic enzymes.

The activity secreted into agar was well below that suggested by the qualitative studies, and, therefore, this procedure was repeated with 4 % Noble Gelatin used in place of agar. The activity was then much greater. This protection afforded the amylolytic enzymes by gelatin may be that of protection from proteolytic enzymes. The scutellar secretion of proteases during germination has been suggested (19) and was readily demonstrable in this work with gelatin by the gradual liquefaction of the gelatin in the area of the implanted scutellum. In agar the secreted proteases would have no substrate other than other secreted enzymes.

Since the scutellum could not be expected to continue its secretion of amylases for many days after being removed from the grain, a system of staggered dissection was utilized as the most satisfactory procedure for determining the amylolytic activity secreted during germination. In this procedure scutella were placed on gelatin for only a 2-day period. Some scutella were germinated intact for 3 days, put on gelatin for the 4th and 5th days, and then the gelatin assayed. Other scutella were germinated intact for 5 days, placed on gelatin for the 6th and 7th days, after which the gelatin was assayed. The sum of the activities of these two assays was assumed to give the total secretion between the 3rd and 7th days of germination. This procedure was carried along to cover the entire germination period. Leaving the germinating axis attached to the scutellum had no effect on the extent of the secretion. Adding soluble starch to the gelatin likewise had no effect. The secretions from 5 scutella were used at each interval assayed.

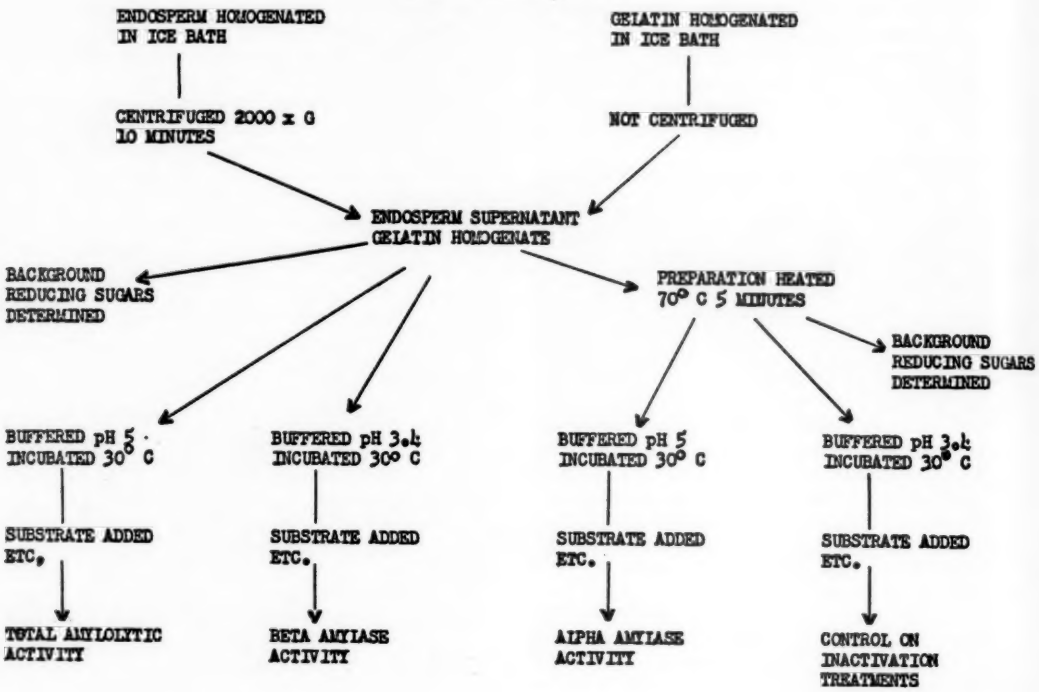
Admittedly, this method is highly artificial and may prove to harbor artifacts. It has been suggested by Hageman (pers. communic.) that maltase may be a contaminant in these preparations of scutellar secretion. This enzyme is present in scutellar particles (11) and may be secreted along with amylase and protease. However, only small amounts of glucose are produced in the endosperm during germination, and this presumably from the slow cleavage of maltotriose to maltose and glucose by alpha amylase (4).

The fact that the secretion could not be measured for the first 3 days of germination is regarded as no handicap to the method used since the total activity in the intact endosperm at 3 days is slight and shown to arise in the endosperm itself (see Results). The gelatin homogenates were not centrifuged since the activity was found to be greater in the very viscous fraction sedimented at 2,000 G than in the supernatant. Otherwise, the assay procedure was the same as described for intact endosperm preparation.

I. SEPARATION OF ALPHA AND BETA AMYLASE. A. *Endosperm of intact seeds.* The endosperm supernatant used to find the total amylolytic activity at intervals during germination was also used in the separate assaying of alpha and beta amylase activity. The separation of the two enzymes involved the selective inactivation of one of the enzymes and assaying for the other, based on differences in their

physical properties (see Flow Sheet). (The physical properties of the amylases from the various cereals are similar (16). Even though these properties have been established chiefly from barley and wheat amylases, they are assumed to hold true for maize amylases.)

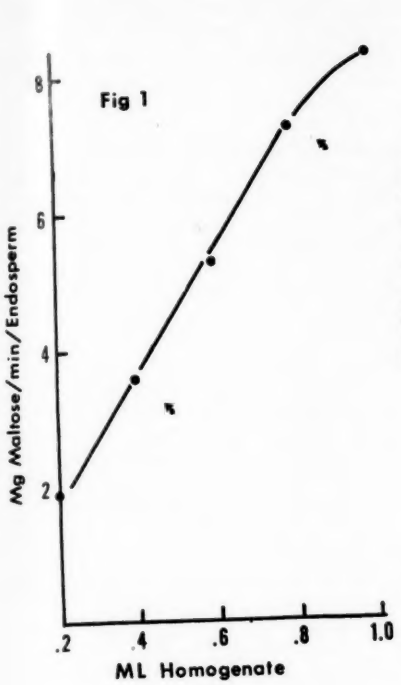
Flow Sheet

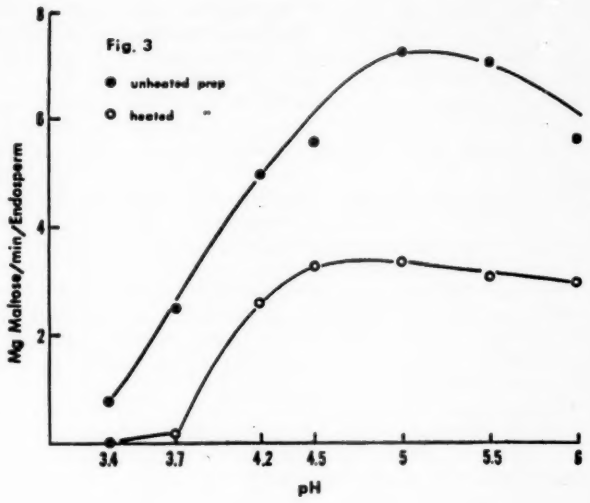

ENDOSPERM HOMOGENATED IN ICE BATH
GELATIN HOMOGENATED IN ICE BATH
CENTRIFUGED 2000 x G 10 MINUTES
NOT CENTRIFUGED
ENDOSPERM SUPERNATANT GELATIN HOMOGENATE
BACKGROUND REDUCING SUGARS DETERMINED
PREPARATION HEATED 70° C 5 MINUTES
BACKGROUND REDUCING SUGARS DETERMINED
BUFFERED pH 5 INCUBATED 30° C
BUFFERED pH 3.4 INCUBATED 30° C
BUFFERED pH 5 INCUBATED 30° C
BUFFERED pH 3.4 INCUBATED 30° C
SUBSTRATE ADDED ETC.
SUBSTRATE ADDED ETC.
SUBSTRATE ADDED ETC.
SUBSTRATE ADDED ETC.
TOTAL AMYLOLYTIC ACTIVITY
BETA AMYLASE ACTIVITY
ALPHA AMYLASE ACTIVITY
CONTROL ON INACTIVATION TREATMENTS

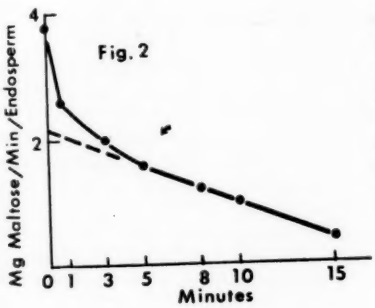
Cereal beta amylase is rapidly inactivated at 70° C, whereas cereal alpha amylase is much more resistant to this temperature (4, 14, 20, 22, 23). Hence, to assay for alpha amylase these preparations were heated to 70° C for 5 minutes and then assayed as previously outlined. Figure 2 shows the loss of activity in an intact endosperm preparation as a function of the time that it is maintained at 70° C. The change in the slope of the curve at 5 minutes is interpreted as indicating the total inactivation of beta amylase by this point. The slope of the curve after 5 minutes is interpreted as indicating the loss of alpha amylase activity, which is more gradual. An interpolation of this latter slope back to zero time suggests that by 5 minutes some loss of alpha activity has also occurred, as well as the complete loss of beta amylase activity.

Cereal beta amylase retains much of its activity at pH 3.4, whereas cereal alpha amylase is completely inactivated at this pH (10, 20, 22, 23). Thus, the assay utilized for the separate determination of beta amylase activity entailed running the previously outlined assay at pH 3.4 The preparations had to be buffered at this pH several minutes before incubation with the substrate to insure the prior inactivation of alpha amylase. A pH curve for the amylolytic activity of an intact endosperm preparation is presented in figure 3 and compared in this figure with the curve for the same preparation heated to 70° C for 5 minutes. The activity of the heated preparation at pH 3.4 is zero, showing that both enzymes have been completely inactivated by their respective inactivation treatments. To serve as a control on these inactivation procedures, some of the heated preparation was assayed at pH 3.4 in every instance in which both alpha and beta amylase were assayed for separately to insure that each treatment had totally inactivated its respective amylase.

The activity of beta amylase is also much reduced at this pH [as much as 50 % in barley preparations (20)]. This, coupled with the indication that some alpha amylase activity is lost in the heat treatment, implies that the sum of the activities of the enzymes







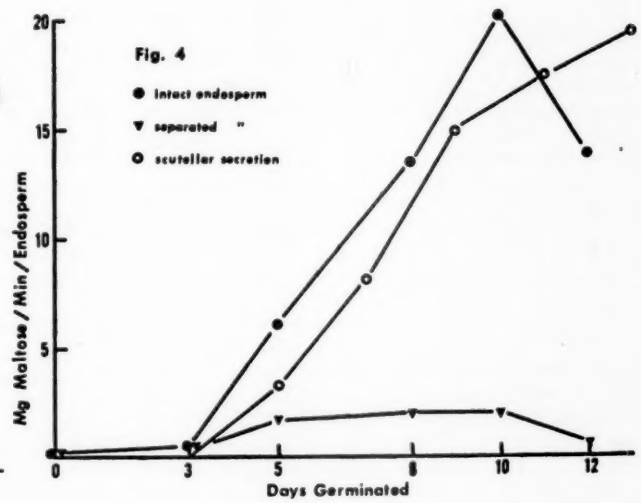


FIG. 1. Substrate saturation curve. Amount of product formed given as a function of the concentration of enzyme (homogenate). Notice that substrate becomes limiting by 1.0 ml of homogenate. Arrows indicate concentrations used in the assays. Assayed at pH 5.0 (6-day intact endosperm preparation).

FIG. 2. Inactivation of beta amylase by 70° C. Dashed line gives theoretical inactivation of alpha amylase. Arrow denotes heating time employed in assays. Assayed at pH 5.0.

FIG. 3. pH Inactivation of alpha amylase. Notice zero activity of heated preparation at pH 3.4 (6-day intact endosperm preparation).

FIG. 4. Total amylolytic activity of intact endosperm, separated endosperm, and scutellar secretion for the germination period. Assayed at pH 5.0

assayed separately would not equal their amylolytic activity assays jointly.

B. *Endosperm separated from embryo.* The same procedure as outlined for intact endosperm was used with the preparations of endosperm from which the embryo had been removed. Since there was no activity in the preparations heated to 70° C in this case (indicating the absence of alpha amylase) the temperature curve does not apply to these preparations.

C. *Scutellar secretion into gelatin.* The same gelatin homogenates as were used to determine the total amylolytic activity of the scutellar secretion were treated as were the endosperm preparations to separately assay for the two enzymes. Since there was no activity found at pH 3.4 in unheated homogenates (indicating the absence of beta amylase in the secretion) the temperature curve does not apply to these preparations either.

Results

Figure 4 shows the total amylolytic activity found A, in the endosperm of intact germinating seeds, B, in the separated endosperm germinated without its embryo, C, secreted during germination by the scutellum. All the activity found in the endosperm during the first 3 days can be accounted for by that found in situ in the endosperm, since the activity in the separated endosperm equals that in the intact endosperm at 3 days. A secretion of amylase from the scutellum cannot be detected during this period. There is a steady increase in the intact endosperm amylase activity and in the secreted activity after the 3rd day. The separated endosperm activity increases somewhat at first but then levels off. There is a marked drop in the activity in the intact endosperm preparations after the 10th day, which may result from the destruction of amylases by the proteases secreted from the scutellum. The nitrogen content of the intact endosperm by the 12th day is less than ⅛ its original value (8), which would suggest the loss of protein that heretofore might have protected the amylases from these enzymes. The decrease in activity in the separated endosperm may possibly result from in situ proteases. The activity secreted from the scutellum, which parallels the increase in activity in the intact endosperm, does not drop off. Rather, its increase begins to level off after the 9th day. It should be noted that this curve of the activity of secreted amylase is compiled from the accumulation of the activity of amylase secreted in 2-day increments. Consequently, the protection afforded by the gelatin from proteases would be very pronounced in comparison with any protection in the intact endosperm during the last stages of germination.

The sum of the activity of secreted amylase and the activity arising in situ in the separated endosperm closely approximate the total activity of the intact endosperm except for the last several days. The data reveal that, although the initial amylase activity in the germinating seed is endosperm amylase, only about 10 % of the total activity at the peak of amylolytic activity (10 days) is due to endosperm amylase; the rest originates in the scutellum.

Figure 5 and table I present a separation of the total amylase activity of the intact endosperm into that attributable to alpha and to beta amylase. In the resting seed and at 3 days after germination commences there is no measurable alpha amylase activity, but thereafter it becomes the dominant amylase, reaching a peak in activity by the 10th day. Beta amylase activity increases considerably from its value in the resting seed, but by the 10th day it is far surpassed by that of alpha amylase.

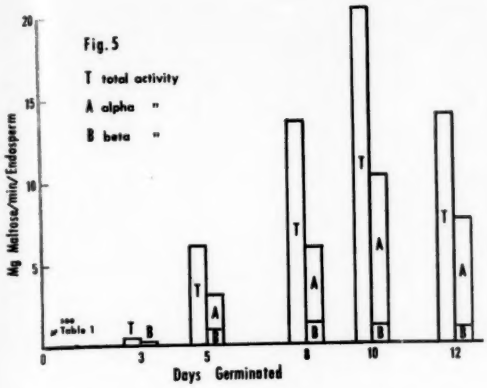
The sum of alpha and beta amylase activities does not come close to equalling the total amylolytic activity of the intact endosperm at any point during germination. The percent that their accumulative activities is of the total activity at any point (table I) averages 53 %. This may be explained by the presence of other starch-hydrolysing enzymes or maltase in the preparations, which are inactivated at pH 3.4 and by the heat treatment and, thus, not active in either of the separate assays, but are responsible for the unaccounted-for activity in the total amylolytic activity assay. But it is more likely that in the selective inactivations of the amylases enough activity is lost from the amylase being assayed in each case to account for this missing activity. The heat treatment appears to decrease alpha amylase activity about 1/3 (fig 2), and the extent of this loss is related to the amount of protein protection present in the preparation (4, 14). In preparations showing no alpha

Table I

Amylase Activity of Intact Endosperm*

	Days germination					
	Resting grain	3	5	8	10	12
Total amylolytic activity	0.10	0.54	6.17	13.60	20.35	14.00
Alpha amylase activity	0.00	0.00	2.16	4.50	9.00	6.40
Beta amylase activity	0.06	0.30	1.06	1.45	1.25	1.13
% Rec.**	60	56	54	44	50	54

* Expressed as mg maltose/minute/endosperm.
** Percentage recovered—percentage of the total activity given by the sum of alpha and beta activities.



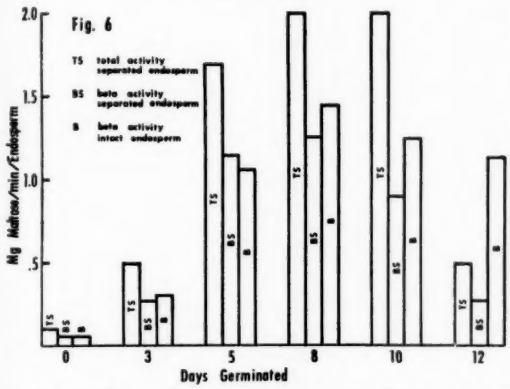


FIG. 5. Separation of the total amylolytic activity of intact endosperm into the activities of alpha and beta amylase. For alpha amylase activity preparations heated to 70° C, assayed at pH 5.0. For beta amylase activity preparations not heated, assayed at pH 3.4.

FIG. 6. Separation of total amylolytic activity of the separated endosperm into that due to beta amylase and compared with the beta amylase activity of the intact endosperm. No alpha amylase activity demonstrable in these preparations. Notice expanded ordinate values. For beta amylase activity preparations not heated, assayed at pH 3.4.

amylase activity (separated endosperm preparations) the beta amylase activity averages only 57 % of the total, suggesting that maize beta amylase may behave like that of barley, which has only 50 % of its maximum activity remaining at pH 3.4 (20). Thus, it may prove legitimate to double the values of beta amylase activity and to consider the figures for alpha amylase to be only 2/3 of their real values, in which case the percent recovery for these intact endosperm preparations would reach 80 %. This percent recovery would make it less likely that there are other enzymes in these preparations catalyzing the hydrolysis of the starch polymer. Pure preparations of maize alpha and beta amylase are necessary before this proposal can be justified or discarded.

Figure 6 and table II give the separation of the total amylolytic activity of the separated endosperm into the activities of the two amylases, and figure 6 also compares the beta amylase activity found in these endosperms with the beta amylase activity of intact endosperms. In these preparations no alpha amylase activity could be detected, but the beta amylase activity does not approximate the total activity. The percentage recovery averages 57 %. Up through the 8th day the beta amylase activity of these preparations roughly agrees with the beta amylase activity of the intact endosperm. This suggests that the beta amylase found in the germinating maize endosperm arises entirely in the endosperm itself. The difference in the beta amylase activities from the two sources after the 8th day may be explained in part by the highly artificial conditions imposed on the endosperm germinating separated from its embryo. The values derived from these preparations may not reflect the intrinsic properties of the endosperm with regard to amylase activity originating in situ. Nevertheless, these preparations do reveal that originating within the endosperm itself there is an increase in beta amylase and no production of alpha amylase during germination. The in situ origin of beta amylase is further borne out by the increase in reducing sugars found as germination progressed in these preparations (table II). The fact that this production of reducing sugars is enzymatic is shown by the fact that there was no increase in reducing sugars over

TABLE II

AMYLASE ACTIVITY OF SEPARATED ENDOSPERM*

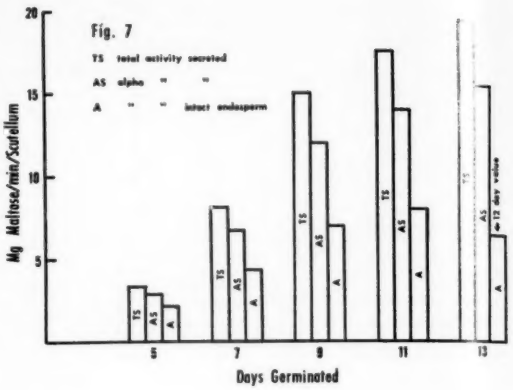
	Days germination					
	Resting grain	3	5	8	10	12
Total amylolytic activity	0.10	0.50	1.70	2.00	2.00	0.50
Alpha amylase activity	0.00	0.00	0.00	0.00	0.00	0.00
Beta amylase activity	0.06	0.27	1.14	1.26	0.90	0.26
% Rec.**	60	54	67	63	45	52
Reducing sugars***	1.68	4.20	5.40	6.60	7.50	8.00

* Expressed as mg maltose/minute/endosperm.
** Percentage recovered—percentage of the total activity given by the sum of alpha and beta activities.
*** Expressed as mg maltose equivalents.

the 12-day period in endosperms that were boiled 2 minutes to serve as a control.

The suggestion that the beta amylase of the germinating seed arises singularly in the endosperm is further supported by the fact that in the separation of the total activity secreted by the scutellum into that attributable to the two amylases (fig 7 and table III) there is no measurable beta amylase activity. The recovery of alpha amylase activity is high, averaging 80 % of the total amylolytic activity of the secretion. This percentage recovery of alpha amylase activity from the gelatin is greater than the percentage recovery of alpha amylase activity from the intact endosperm preparations. This protection from heat inactivation of alpha amylase by gelatin is in line with the findings of other workers (4, 14) who ascribe the heat resistance of the enzyme to the presence of other protein in crude preparations. In fact, crystalline alpha amylase from barley malt is inactivated at 37° C (4). This high recovery of alpha amylase activity in the gelatin preparations and the absence of any demonstrable beta amylase activity suggests that all the secreted amylolytic activity is due to alpha amylase. That some of the reducing power of the secretion preparations might be due to maltase has not been ruled out, however.

In figure 8 the accumulated activity of secreted amylase is compared with the amount secreted in each 2-day period. There is no detectable secretion during the first 3 days, but by the 5th day considerable activity is found. The peak of secretion occurs in the 7 to 9-day period, after which it drops off markedly.



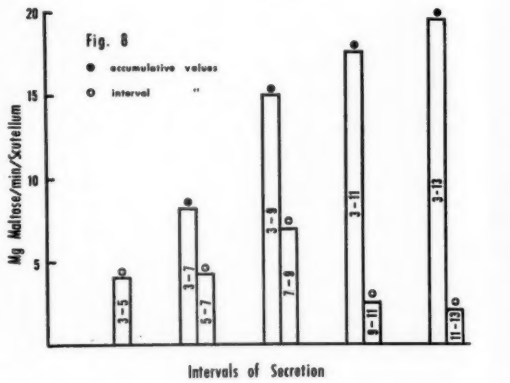


FIG. 7. Separation of the total amylolytic activity of the scutellar secretion into that due to alpha amylase, and compared with the alpha amylase activity of the intact endosperm. No beta amylase activity demonstrable in these preparations. For alpha amylase activity preparations heated to 70° C, assayed at pH 5.0.

FIG. 8. Separation of the total amylolytic activity of the scutellar secretion into the amounts secreted in 2-day increments.

DISCUSSION

As can be seen from the data, there is very little amylolytic activity in the endosperm of the dormant maize kernel, and all of this activity is attributable to beta amylase, two findings in agreement with numerous workers (4, 15, 20, 24, 26). During germination both alpha and beta amylase activities increase in the endosperm, but alpha amylase becomes by far the chief amylolytic enzyme, another finding supported

TABLE III

AMYLASE ACTIVITY SECRETED FROM SCUTELLUM INTO GELATIN*

	DAYS GERMINATION									
	0–3	3–5	5–7	3–7	7–9	3–9	9–11	3–11	11–13	3–13
Total	0.0	4.0	4.2		6.9		2.5		2.0	
Alpha		3.5	3.5		5.2		2.0		1.4	
Beta		0.0	0.0		0.0		0.0		0.0	
% Rec.**		88	89		75		80		70	
Total				8.2		15.1		17.6		19.6
Alpha				6.8		12.0		14.0		15.4
Beta				0.0		0.0		0.0		0.0

* Expressed as mg maltose/minute/scutellum.
** Percentage recovered—percentage of total activity given by sum of alpha and beta activities.

by many other workers (7, 15, 24, 25). The apparent separate and exclusive origin of each enzyme —alpha amylase in the scutellum, and beta amylase in the endosperm—shows the endosperm to have some independent autodigestive capability, but shows that the chief tissue in bringing about the mobilization of the endosperm starch in maize germination is the scutellum. There is very little alpha amylase activity in the scutellum of the resting grain (unpubl. data), and whether or not the increase of this enzyme during germination represents a net synthesis of enzyme in this tissue is not clear. A net synthesis of this enzyme during germination has been reported for barley seeds (10), but the activity of this enzyme in many cereal malts can be increased by treatment with papain (20, 30), which implies the freeing of the enzyme from a bound form. Maize behaves similarly to barley with respect to the time of appearance of alpha amylase. In barley this enzyme first appears on the 3rd day of germination and increases through the 8th (4). The great increase in alpha amylase activity is also characteristic of germinating wheat where a 6,000-fold increase in the activity of this enzyme has been reported (14).

The relatively slight increase in beta amylase activity during germination is characteristic of maize and some other cereals (24). The activity of this enzyme during the initial stages of germination can be artificially increased to its maximum activity in germination in barley by treating malts with proteases also. This has caused many workers to doubt a net synthesis of this enzyme during germination in many cereals (10, 20). This appears reasonable in light of the data which place the origin of this enzyme singularly in the endosperm, a tissue which is shown more and more to be metabolically inert after the seed reaches maturity.

Summary

The extent of the activities of alpha and beta amylase in the endosperm of germinating maize was followed through the germination period. Also the amount of each enzyme secreted by the scutellum and arising in situ in the endosperm during germination was followed. The results revealed:

I. Alpha amylase originates exclusively in the scutellum and is secreted during germination.

II. Alpha amylase accounts for 9/10 of the amylolytic activity found in the endosperm at the peak of amylolytic activity 10 days after germination has begun.

III. An interaction between the scutellum and the endosperm must take place before the scutellum will secrete alpha amylase into the endosperm.

IV. Beta amylase is the only amylase in the endosperm of the resting seed.

V. Beta amylase is formed exclusively in situ in the endosperm during germination.

VI. Beta amylase accounts for only 1/10 of the total amylolytic activity in the endosperm at the peak of amylolytic activity during germination.

The methods used in capturing the amylase secreted from the scutellum, in determining the amylase arising in situ in the endosperm, and in separately assaying for the two enzymes are given and the results are discussed in the light of what is known concerning the amylases in cereal germination.

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EFFECTS OF INDOLEACETIC ACID AND KINETIN ON SCOPOLETIN-SCOPOLIN LEVELS IN RELATION TO GROWTH OF TOBACCO TISSUES IN VITRO[1, 2]

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The levels of both scopoletin and its glycoside, scopolin, in tobacco tissues cultured in vitro have been shown to be markedly influenced by the supply of 3-indoleacetic acid (IAA) and kinetin in the nutrient medium, and these substances likewise influence the release of scopoletin to the medium (15). A more detailed quantitative study has, therefore, been carried out to determine possible causal relationships between the actions of IAA and kinetin in these phenomena and the effects of these chemicals on growth and differentiation of the tissues.

MATERIALS AND METHODS

In the earlier work scopoletin was extracted from the agar medium. In order to measure the concentration of scopoletin in the medium directly by fluorimetry a liquid culture technique similar to one described by Heller (10) has been adopted. A 19 × 50 mm Pyrex test tube containing 10 ml of modified White's medium (14) less agar, was used for the culture of a single explant. The tissue was supported on a filter pad. A 5.5 cm disc of Whatman No. 2 filter paper suitably folded to form a cylinder with one end open, was inserted, open end first, into the tube and the pad so formed pushed down until its surface was just level with that of the medium.

Nicotiana tabacum L. (var. Wisconsin #38) tissues were used throughout these studies. Pith sections of approximately 65 mg fresh weight were prepared according to (12) and callus explants of approximately 20 mg fresh weight were cut from stock tissue grown in Erlenmeyer flasks on agar medium containing 2 mg/l IAA and 0.2 mg/l kinetin. The tissues were cultured from 2.5 to 24 days. Except as stated the tubes were kept in batches on shelves exposed to high (ca. 80 %) relative humidity and continuous weak light from fluorescent ceiling fixtures. The room temperature was 28° C. In all experiments ten replicate cultures were pooled for fresh and dry weight determinations. The media were combined, made up to 100 ml with distilled water and assayed fluorometrically.

For each determination of scopoletin and glyco-

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side in the tissue approximately 250 mg samples of fresh tissue were frozen at −15° C and homogenized with 10 ml of 80 % ethanol in a Potter-Elvehjem homogenizer. The homogenate was centrifuged, the supernatant decanted, and the procedure repeated twice with 5 ml volumes of 80 % ethanol. The last supernatant showed little fluorescence under ultra violet light. The three supernatants were pooled and evaporated to dryness in a 30 ml beaker in a stream of air. The residue was then dissolved in about one milliliter of 80 % ethanol and applied to an 11 cm starting line of a Whatman 3 MM paper chromatogram. The chromatogram was developed in *n*-butanol : acetic acid : water, 4 : 1 : 2 v/v, mixed immediately before use. Development was allowed to proceed overnight and stopped when the front had moved about 40 cm from the origin. Under these conditions scopoletin and the glycoside separated as two brightly fluorescing bands which, after drying, were cut out and eluted with 0.14 M boric acid buffer adjusted to pH 10.0 with sodium hydroxide. Each eluate was made up to 5.0 ml and its fluorescence measured. The photofluorometer was a Coleman 12C fitted with Corning 5874 primary and 3389–4308 secondary filters and adapted to carry 10 × 77 mm Pyrex test tubes as cuvettes. With each series of measurements a sample of scopoletin (2 μg) was chromatographed, eluted, and used to calibrate the instrument. In measurements of scopoletin directly in the medium the standard sample was dissolved in fresh nutrient solution. The standard scopoletin had been prepared in a crystalline form from hydrolyzed tobacco root extract by a method similar to that of Andreae (1). The fluorescence of the glycoside as such was used for its determination. In pH 10.0 borate buffer the intensity of fluorescence of the glycoside was found to be only 0.048 that of the scopoletin released from it on hydrolysis and determined in the same medium. All steps in the extraction, separation, and determination of these photolabile compounds were carried out in darkness or in weak diffuse light.

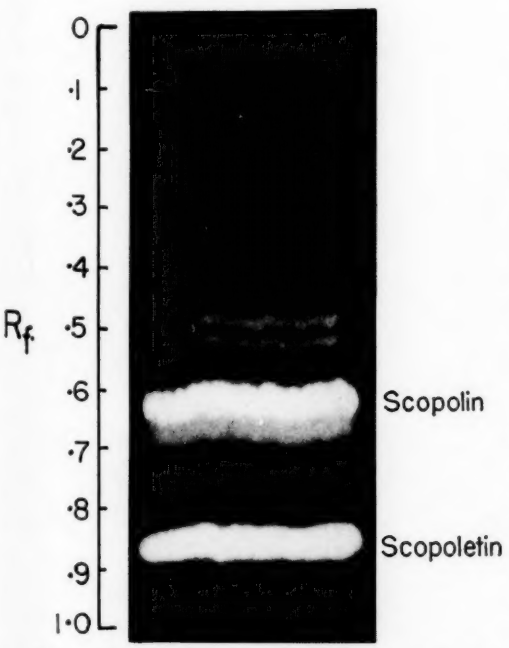


FIG. 1. Paper chromatogram of ethanolic extract of tobacco callus photographed under ultraviolet light. Paper: Whatman No. 3 MM. Solvent: *n*-butanol : acetic : acid : water = 4 : 1 : 2 v/v. Direction: descending.

RESULTS

A typical separation of fluorescent components obtained by chromatography of an ethanolic extract is shown in figure 1. The chromatogram was photographed under ultra violet light. (u.v.) Analysis of the glycoside has shown it to be scopolin. In the butanol : acetic : water solvent it separated as a distinct band at R_f 0.61, while scopoletin ran farther ahead at R_f 0.85. When conditions of culture favored the formation of chlorogenic acid in the tissue (21) this compound was found to run close to scopolin, at R_f 0.67. A number of less intense bands running slower than scopolin have been analysed and shown to be glycosides of scopoletin. Details of analysis of these and of scopolin will be reported in a later paper.

The fresh weight and scopoletin status of a series of pith sections cultured for 24 days in IAA and kinetin concentration ranges of 1 to 100 mg/l and 0.02 to 2.0 mg/l, respectively, are shown in figure 2. Dry weight increases closely paralleled those of fresh weight, and only the latter are shown in figure 2A. From the data it is clear that vigorous growth is limited to a rather narrow range of IAA concentrations (around 1 mg/l) and that kinetin exerts a marked growth promoting effect in the presence of this concentration of IAA. Kinetin alone does not promote growth of the pith tissue. The relatively quiescent cells of the pith as removed from the tobacco stem are low in both scopolin (equivalent to 14.9 μg/G.F.W. scopoletin) and scopoletin (0.27 μg/G.F.W.) and begin to produce these substances in quantity only when cultured under conditions which lead to tissue growth. The striking feature of the data shown in figure 2B is the rapid rise in scopolin content of the tissue as the exogenous supply of kinetin is increased. This rise is paralleled by one in the endogenous scopoletin content of the tissue suggesting a state of equilibrium between the aglycone and its glycoside, but a comparison of the ordinate scales of figures 2B and 2C (ratio 13 : 1) shows this equilibrium to be strongly towards the latter. Under conditions of maximal growth (1 mg/l IAA and 0.2 mg/l kinetin in this case) a depression in the curve relating scopolin concentration with kinetin supply was consistently observed. This low level of scopolin could not be correlated with a rise in the endogenous

scopoletin level (a depression was likewise observed), neither was there an increase in the amount of scopoletin released to the medium under these conditions. It should be emphasized that here, as in earlier studies (15), scopolin was never detected in the external medium.

The free scopoletin released to the medium (figure 2D) is expressed in terms of fresh weight of the tissue rather than as concentration in the medium. In general very low external scopoletin levels are associated with vigorously growing tissue, and it will become clear that scopoletin is lost only by cells in an abnormal or dead state.

Two other points are of interest. First, high, yet not toxic IAA concentrations shift the scopolin/scopoletin equilibrium somewhat toward scopoletin; and second, in the absence of kinetin and with scopolin formation at a minimum, relatively high levels of scopoletin are found both within the tissue and in the external solution.

The above experiment was repeated under identical conditions except that callus tissue was substi-

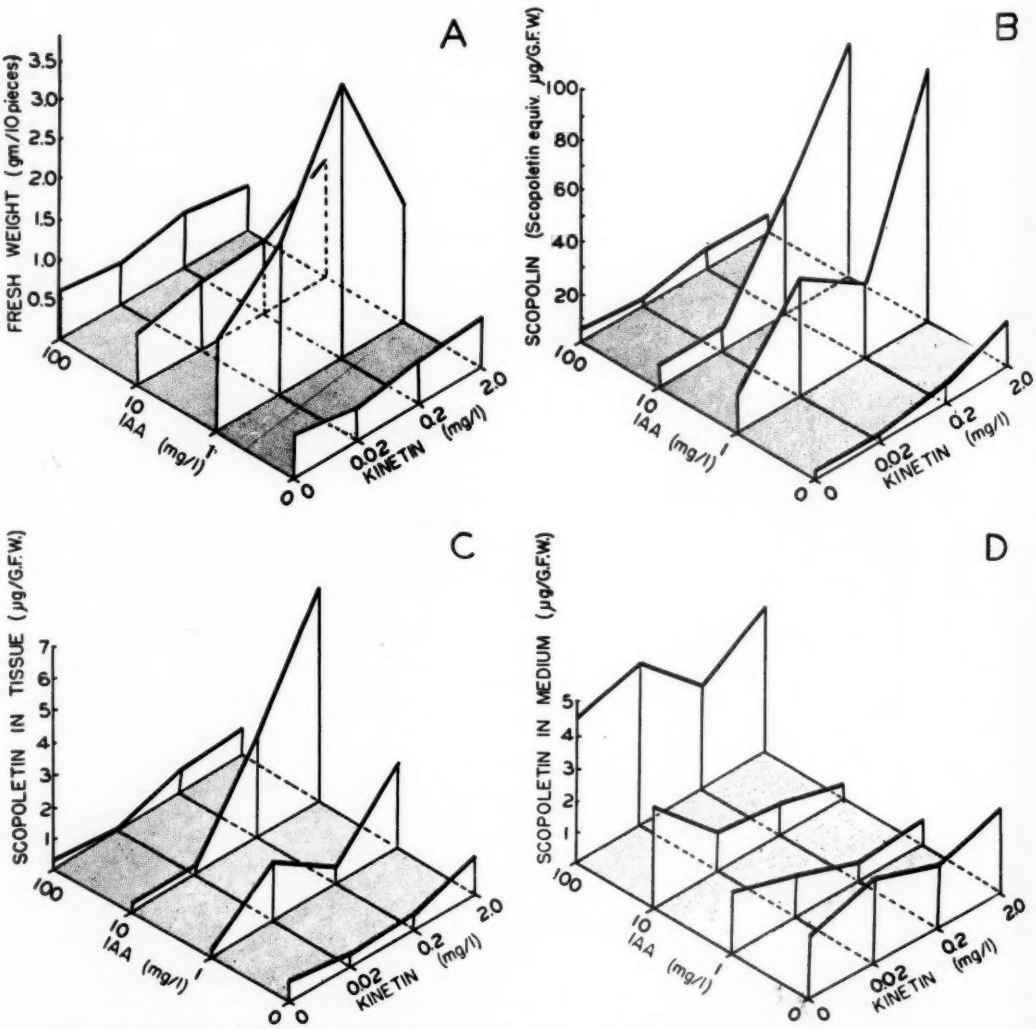


FIG. 2. Effect of IAA and kinetin on the fresh weight and scopolin and scopoletin levels in pith tissue after 24 days. A: Fresh weight of tissue (Dry weight = 4–7 % of fresh weight). B: Scopolin level in the tissue. C: Scopoletin level in the tissue. D: Scopoletin released to the medium. Experiment started 9 January 1959. Original fresh weight ca. 0.65 g/10 pieces.

tuted for pith and sampling was done at 3, 6, 12, and 24 days after the cultures were started. This experiment was performed three times, and, although there was some variation between experiments, the influence of IAA and kinetin on the scopoletin status of this tissue was marked, consistent, and parallel to that observed in pith. Representative results obtained after 3 and 24 days in one experiment are shown in figures 3 and 4, respectively.

In comparing figure 4 with figure 2 it is immediately obvious that scopolin which is produced in relatively small amounts in pith in the absence of exogenous IAA, occurs in high concentrations in callus also when the auxin is not supplied. This no doubt reflects the relatively greater ability of the callus tissue to produce its own auxin. It is also clear that in callus, kinetin becomes inhibitory to growth at a lower concentration than in pith. When these characteristic differences between the two tissues are considered it is apparent that the changes in scopoletin status associated with the supply of IAA and kinetin in pith occur also in callus.

The excision of explants from stock callus pieces damages many cells at the cut surfaces. Associated with such wounding is a rapid rise in the scopoletin level of the tissue, no doubt through enzymatic hy-

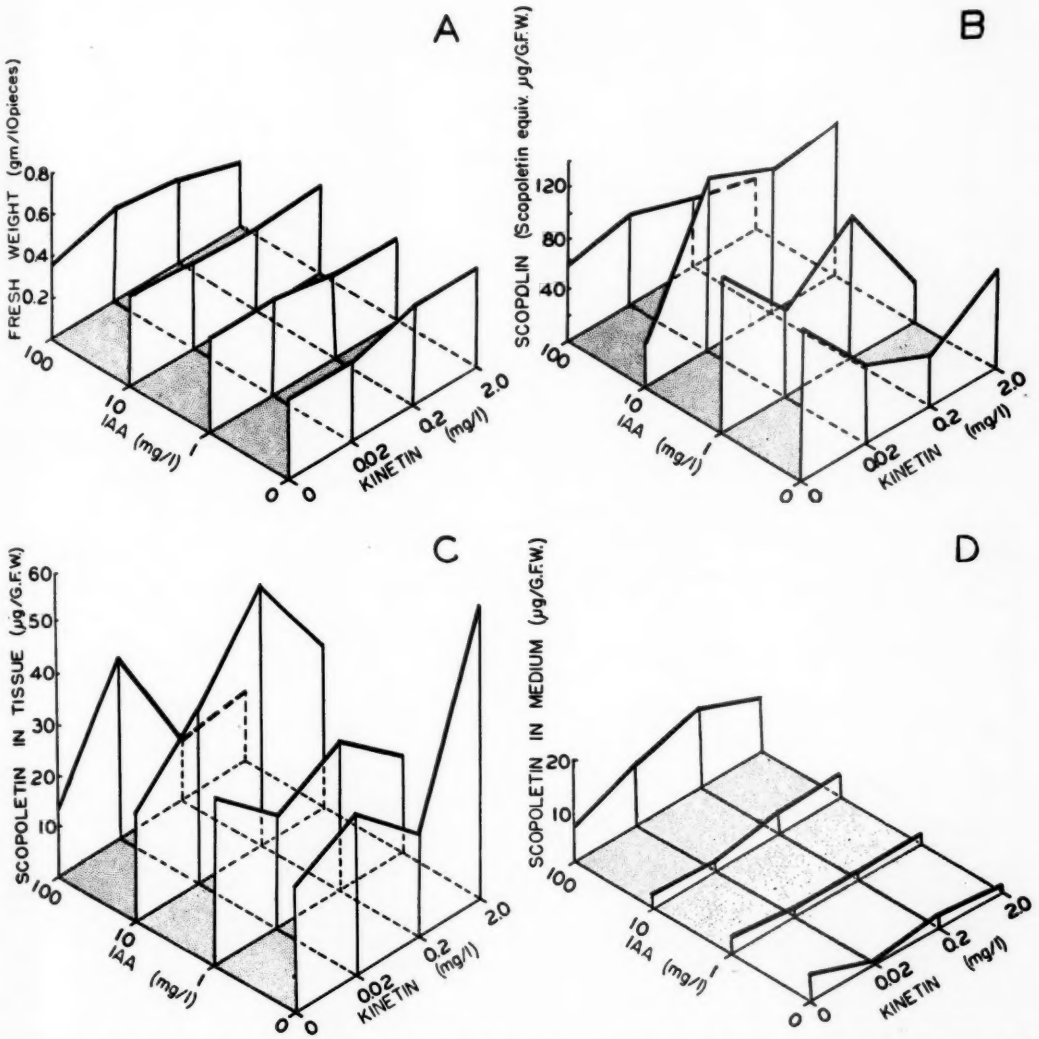


FIG. 3. Effect of IAA and kinetin on the fresh weight and scopolin and scopoletin levels in callus tissue after 3 days. A, B, C, and D, as in figure 2. Experiment started 7 February 1958. Original fresh weight ca. 0.30 g/10 pieces.

drolysis of scopolin. This generally high level of scopoletin still exists after 3 days (figure 3C) but already at this time when little growth differential is apparent, changes are beginning to occur in the levels of scopolin in response to the different treatments (figure 3B). Moreover, the scopoletin which is maintained in equilibrium with the scopolin in the tissue contributes to the pattern of total endogenous scopoletin, shown in fig 3C. A loss of scopoletin to the medium following the supply of toxic concentrations of IAA to the tissue is clear from the effect of 100 mg/l IAA, in figure 3D.

Analyses of the 6 and 12 day samples showed a general reduction in the level of endogenous scopoletin along with a development of the differential growth response and scopolin and scopoletin levels observed after 24 days and illustrated in figure 4. The high levels of scopoletin in the medium in the absence of kinetin after 24 days was preceded by a high level of scopoletin in the tissue after 12 days.

Further evidence for the interpretation that scopolin is degraded to scopoletin and that this is then released to the medium as a result of injury to the tissue or physiologically unfavorable conditions is

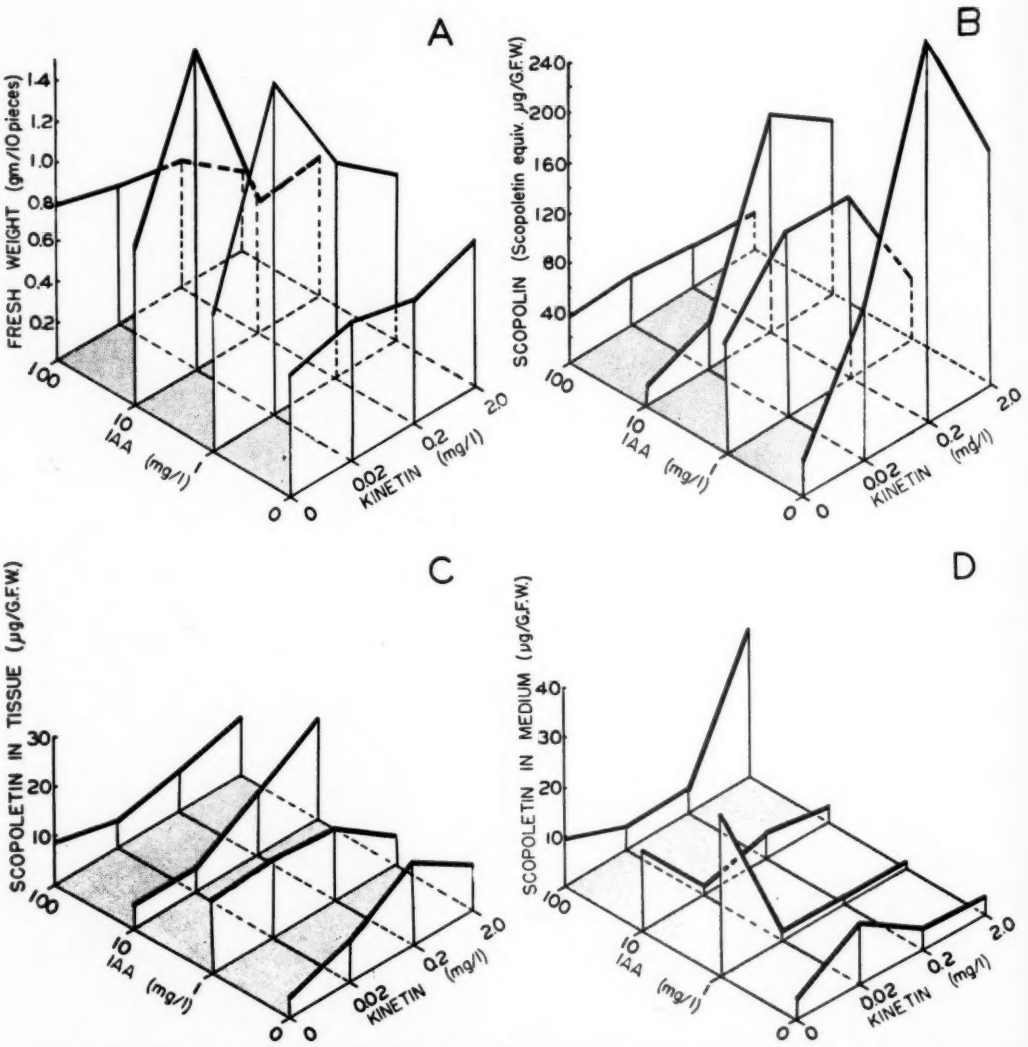


FIG. 4. Effect of IAA and kinetin on the fresh weight and scopolin and scopoletin levels in callus tissue after 24 days. A, B, C, and D, as in figure 2. Experiment started 7 February 1958. Original fresh weight ca. 0.30 g/10 pieces.

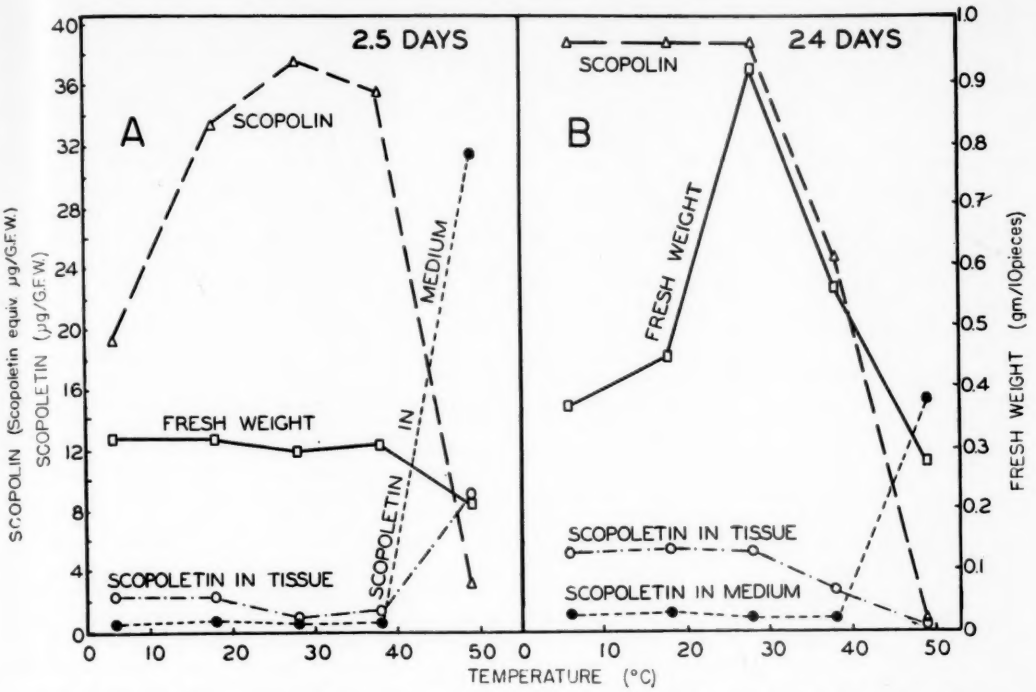


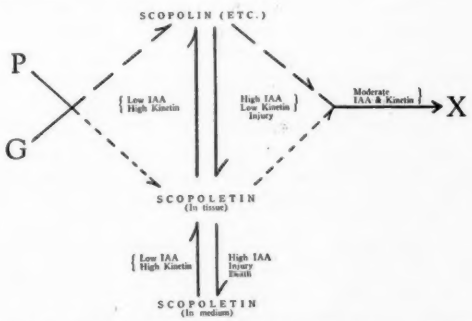
FIG. 5. Effect of temperature on the fresh weight and scopolin and scopoletin levels in callus tissue. A: After 2.5 days. B: After 25 days. Experiment started 7 January 1959. Original fresh weight ca. 0.30 g/10 pieces.

afforded by two experiments in which callus was grown at different temperatures on a medium favorable for growth. The temperatures ranged from 6 to 49° C as shown in figure 5. In one experiment (fig 5A) the tissues were harvested after 2.5 days, in the other (fig 5B) after 24 days. It is clear from both figures that as the temperature increased from 6 to 38° C the level of scopoletin in the medium remained unchanged. However, at 49° C a very high external level had been attained, strongly indicating that the release of scopoletin from the tissue is due to the death of component cells. After both time intervals the level of scopolin in the tissue was observed to fall as the temperature rose from 28 to 49° C. In the 24 day old cultures, this fall was paralleled by a drop in scopoletin level in the tissue. In the young cultures (2.5 days) there was actually a rise in the level of free scopoletin which was almost certainly the result of degradation of scopolin under conditions unfavorable for growth.

Also when toxic concentrations of cyanide, coumarin, maleic hydrazide, ascorbic acid, or triiodobenzoic acid were supplied to the callus a sharp increase was observed in the release of scopoletin to the medium.

DISCUSSION

A diagram is presented to summarize possible influences of auxin and kinetin levels and proportions on quantitative relationships between scopoletin and scopolin and between these substances and their close metabolic relatives.



The formation of scopolin from a precursor, P, and glucose or a glucose derivative, G it is suggested, is promoted as a function of the concentration of added kinetin. The question is left open whether

the synthesis proceeds via scopoletin or some other pathway. It is clear, however, from unpublished work (Sargent and Skoog) that other carbohydrates may substitute for glucose to form a variety of scopoletin glycosides (several are evident as fluorescent bands in figure 1). The level of scopolin attained in the tissue is also dependent on the auxin content. A minimal level of auxin of either endogenous or external origin would seem to be required for the accumulation of very high levels of scopolin, but as the concentration of added IAA is increased the scopolin level falls drastically. With moderate, growth-promoting concentrations of IAA, scopolin is converted in large part to some non-fluorescent product, X, which presumably is utilized in cellular synthesis; likely in lignin formation. Again the question is left open whether or not this transformation occurs via scopoletin as an intermediate. Another possibility would be that scopoletin merely is set free in the process. In any case some free scopoletin is present in the tissue, and as the IAA level is raised, higher amounts appear. With toxic levels of IAA or under otherwise unfavorable conditions for growth much scopoletin is released to the external medium. In such cases, it is proposed, the free scopoletin in the tissues and medium definitely is derived at least for the most part from degradation of scopolin and/or other scopoletin glycosides which would normally have been utilized in growth.

The reverse process, i.e., the incorporation of exogenous scopoletin, has not been studied in detail here, but a marked growth effect of scopoletin added together with kinetin has been observed in tobacco callus cultures (15). This is evidence that scopoletin is in fact assimilated, and suggests that the process requires a kinin; at least it is promoted by kinetin.

In the above diagram kinetin-IAA levels are indicated to affect the direction of equilibrium reactions between scopoletin and its different combined forms or derivatives. In view of the time relations in the present experiments, it is of course likely that steady states of synthesis and degradation rather than true, reversible equilibrium reactions are being observed. It should be pointed out that not only do the levels of scopoletin and scopolin vary, but also the fluorescent bands of other scopoletin glycosides, shown in figure 1, change in relative intensities with variations in the kinetin and IAA supplies in the medium.

It is clear, therefore, that complex quantitative relationships exist between free scopoletin and its several bound forms which are intimately dependent on the availability, levels, and proportions, of these growth substances.

A large number of plants are known to accumulate scopoletin especially under conditions unfavorable for growth (8). Best (3, 4, 5) who observed an increase in scopoletin content of tobacco plants following injury or viral infection put forward a number of hypotheses to explain this, but he favored one which postulated the diversion of normal metabolism resulting in scopoletin accumulation. The path, P → scopolin → X, in the scheme presented here as the major one in healthy rapidly growing tobacco tissue would be in accord with this and with Best's observations that the level of scopoletin in healthy tobacco plants is minimal when the growth rate is maximal. An accumulation of a blue-fluorescing material (presumably scopoletin) around mechanical cracks or around lesions induced by boron deficiency in celery has been recorded by Spurr (18). That viral infection per se did not result in scopoletin formation in potato tubers (1) but that physical damage to the cells of the tuber was necessary has been shown by Sanford and Grimble (17). Andreae working with a variety of potatoes and Best with Nasturtium could not detect scopoletin (or fluorescence) in these plants prior to injury. It may be assumed, therefore, that these plants also exhibit a rapid normal turnover of glycoside to X. Of interest in this connection was the finding of Andreae and Andreae (2) that the fluorescence observed in the deformed portions of potato leaves infected with leaf-roll virus was associated with a local accumulation of starch. From physical properties it appears certain that the scopoletin glycoside reported in Avena roots is identical with the scopolin obtained from tobacco (9, 7). Also, in potato tubers the presence of scopolin seems likely from chromatographic properties of fluorescent substances extracted by Burton (6); and Housley and Taylor (11) have recently claimed that scopoletin is a component of inhibitor β extracted from the tubers. The role of scopoletin as a natural growth regulator in roots has been studied by Goodwin and his students (16) and in tissue cultures as a substitute for IAA by Montaldi and Skoog (15). No determination of the effect of scopolin on growth has been reported, but it is of interest that the root-inhibiting properties of esculetin are not shared by its glucoside esculin. Pertinent to this is the recent finding by Towers et al (19) that maleic hydrazide supplied to wheat leaf segments becomes detoxified by the formation of a β-glycoside. Finally, Johnson and Fults (13) have demonstrated the formation of high concentrations of free scopoletin in a number of plants which they treated with high concentrations of 2,4-dichlorophenoxyacetic acid and have suggested this as a possible cause of the herbicidal effect of the chemicals. (20). Evidence derived from diverse sources, therefore, points to the participation of a scopoletin system associated with normal plant anabolism, to possible disruption of this system under conditions of disease or injury, and to its apparent control by growth substances. Although the exact composition, interconversion and other relationships of many of its components, including unidentified fluorescent intermediates and the postulated P and X still remain to be determined, at least it is clear at this stage that the scopoletin status within tobacco tissue is markedly influenced by the supply of IAA and kinetin and, furthermore, that the functioning of scopoletin in the growth process must include the participation of its glycosides and their derivatives.

Summary

The levels of scopoletin and its glucoside, scopolin, have been investigated in both tobacco pith and tobacco callus grown in vitro and supplied with various concentrations of IAA and kinetin over a period of 24 days. The amount of scopoletin released to the medium under these conditions has also been determined. The effect of temperature on these levels has been observed. It is concluded that the formation of scopolin by the tissue is dependent upon a supply of kinetin and that a steady state equilibrium exists between this glycoside and its aglycone. In healthy tissue the scopolin : scolopetin ratio is extremely high, a value of ca. 13 : 1 was observed. Under conditions which favor growth, scopolin is converted to a non-fluorescent substance (X) presumed to be utilized in the growth process. Degradation to scopoletin is accelerated by a high supply of IAA or injury to the tissues. Death or abnormally functioning cells result in release of scopoletin to the medium. The data are discussed in relation to earlier findings.

Acknowledgment

Laboratory assistance by Mr. Minh D. Nguyen is appreciated.

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RESPIRATION AND PHOSPHORYLATION OF MITOCHONDRIA FROM NORMAL AND CROWN-GALL TISSUE CULTURES OF TOMATO[1, 2]

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Knowledge of the initiation mechanism of the crown-gall disease incited by *Agrobacterium tumefaciens* (Smith & Town.) and the subsequent growth of the abnormal tissue is fundamental to understanding diseased growth (2, 18, 24). The nutritional requirements for normal and gall tissues have been intensively studied (25), and the necessity for proper balance of amino acids and growth substances in the gall formation has been emphasized (2, 23). Braun (3) has suggested that conversion of a normal cell into a crown-gall cell is accompanied by the permanent activation of a series of growth-substance-synthesizing systems, which are precisely regulated in all normal cells. Similarly, Klein (17) has proposed stepwise activation of metabolic systems, such as respiration as well as protein and nucleic acid syntheses, during the period of transformation of a normal cell into a tumor cell.

Biochemical analyses are needed to clarify further the metabolic characteristics of the diseased growth. Experiments are especially interesting with isolated enzymes or enzyme systems from normal and diseased tissues. Recent results with plant tissue cultures in studies of cell cytology and physiology (16), and pathology (12, 36) have made attractive the use of such cultures for biochemical analyses.

Earlier respiratory studies of normal and crown-gall tissues were concerned largely with tissue slices or suspensions of intact cells from tissue cultures at various stages of growth. In general, reduced respiratory levels were observed with crown-gall tissue (24). The results, however, were not always consistent; they were dependent upon the basis for expressing the respiratory activity (2).

The purpose of the present paper is to report the oxidative and phosphorylative properties of mitochondria isolated, respectively, from normal and crown-gall tissue cultures of tomato. Portions of the work already have been summarized (31, 33).

MATERIALS AND METHODS

TISSUE CULTURES: The tissue cultures were originally isolated from normal stem or crown gall on stems of tomato plants (*Lycopersicum esculentum* Mill var. Bonny Best). They had been cultivated for 5 years on a White's agar medium (35) modified by Hildebrandt et al (13), and supplemented with 2,4-dichlorophenoxyacetic acid and coconut milk (29). Both normal and crown-gall tissue cultures grew at similar rates on the above medium. Normal tissue cultures were unable to grow in the absence of coconut milk, whereas crown-gall tissue cultures maintained growth at a reduced rate (30).

PREPARATION OF MITOCHONDRIA: Tissue cultures 15 to 20 days old were used as a source of mitochondria. During this period, tissues were actively growing and had a relatively constant respiratory level [avg Qo_2 (N), 80] and respiratory quotient (avg R.Q., 0.9) (30). About 50 g of tissue were washed in 1 liter of M/200 phosphate buffer, pH 7.4, for a few minutes to remove agar, salts and other components of the medium which might be on the surface of the cells. The suspension of cells was then filtered on four layers of cheesecloth. Any necrotic tissue was removed. The washed cells were ground in a glass homogenizer with a Teflon pestle in a solution containing 0.5 M mannitol, 0.005 M cysteine, 0.02 M KH_2PO_4 and sufficient tris-hydroxymethylaminomethane (tris) to bring the pH to 7.4. The homogenate was centrifuged at 500 G for 10 minutes to remove cell debris. The supernatant was centrifuged further at 5,000 G for 10 minutes, and the pellet was suspended in 0.4 M mannitol of pH 7.8 adjusted with tris. The suspension again was centrifuged first at low and then at high speeds. The final pellet from the high speed centrifugation was resuspended in a small amount of the 0.4 M mannitol, and this suspension was used as the mitochondrial preparation in the following experiments.

A high percentage of particles in the preparation was similar in appearance to mitochondrial particles seen with phase optics in living tomato cells in cul-

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ture. These particles were dark-brown, spherical bodies that ranged in size from 0.5 to 2 μ. No morphological differences were observed between mitochondrial particles from the normal and crown-gall tissue cultures. With a fraction spun down at 12,000 G for 10 minutes in 0.5 M sucrose solution, roughly half of the particles stained with Janus green B.

DETERMINATION OF OXYGEN CONSUMPTION: Oxygen consumption was measured with an oxygen electrode apparatus (9) which consisted of a rotating, polarizing, platinum electrode and a recording millivoltmeter. The change in concentration of oxygen in the reaction solution was detected as the change in the cathode reaction current which was converted into voltage and recorded automatically. The reaction cells used (9) were of 2.0 to 2.5 ml capacity with two narrow necks, one for inserting the electrode and the other for adding reaction components and subsequent stirring with a glass rod. Normally the reaction took place with the following amounts of materials per ml of the reaction mixture: 5.0 μmoles of inorganic orthophosphate, 0.5 μmoles of ethylenediamine tetraacetic acid, 5.0 μmoles of $MgCl_2$, 10.0 μmoles of KCl, 0.25 μmoles of mannitol, 2.5 μmoles of substrate and a mitochondrial suspension that contained 130 to 200 μg protein nitrogen. The concentration of oxygen in the air-saturated, isotonic medium was regarded as 240 mμ M at 26° C (6).

DETERMINATION OF PHOSPHORYLATION: The reaction mixture contained, in addition to the basal components mentioned above, radioactive inorganic orthophosphate (60,000 to 100,000 cpm/ml), adenosine triphosphate (ATP, 1 μmole/ml) and sufficient hexokinase and glucose. An aliquot of 0.1 ml of the reaction mixture was withdrawn soon after adding the mixture of ATP, glucose and hexokinase. The second and third 0.1 ml aliquots were taken at the end of the reaction after all oxygen in the reaction mixture had been consumed. Esterified P^{32} in each aliquot was analyzed according to the chromatographic method of Hagihara and Lardy (10). The oxygen consumption between the first and second (or third) samplings was usually 300 to 400 mμatoms, and the time elapsed was 3 to 6 minutes. From a few reaction mixtures with a low rate of respiration, the second and third aliquots were taken before oxygen consumption was completed to avoid introducing an error from air diffusion.

PREPARATION OF ASCORBIC ACID OXIDASE: Ascorbic acid oxidase was extracted from the cell debris fraction of crown-gall tissue cultures by treatment with 0.8 M $(NH_4)_2SO_4$ (pH 7.0 with NH_4OH) for 12 hours at 5° C. After extraction the cell debris was removed by centrifugation. Ammonium sulfate was added to the supernatant until the concentration was 1.6 M. The precipitate was discarded. The soluble fraction was dialyzed against M/15 phosphate buffer, pH 7.0, and then used as the ascorbic acid oxidase preparation.

RESULTS

Typical respiratory responses of mitochondria from normal and crown-gall tissue cultures to various substrates, to cofactors and to inhibitors as traced by the oxygen electrode apparatus are illustrated in figure 1 with the same right to left direction as the record sheet. Starting at the upper right edge, the oxygen consumption by the endogenous respiration of the preparations was 0.8 mμatoms of oxygen/minute/ml of the reaction mixture. Upon addition of succinate, the rate was increased to 13.6 and 11.7 mμatoms, respectively, for normal and crown-gall particles. These rates were accelerated 2.4- and 1.4-fold, respectively, by adding adenosine diphosphate (ADP). Malonate inhibited the succinate oxidation completely. Oxidation of citrate occurred in the presence of malonate and was enhanced by adding diphosphopyridine nucleotide (DPN). Reduced diphosphopyridine nucleotide (DPNH) was oxidized rapidly, as indicated by a sharp change in slope of the curves after its addition. Antimycin A strongly inhibited DPNH oxidation, whereas ascorbate was oxidized rapidly in the presence of Antimycin A.

Figure 1 shows that the mitochondrial preparations from normal tissue cultures oxidized succinate, citrate and DPNH more rapidly, but ascorbate more slowly, than the mitochondrial preparations from crown-gall tissue cultures, as discussed later.

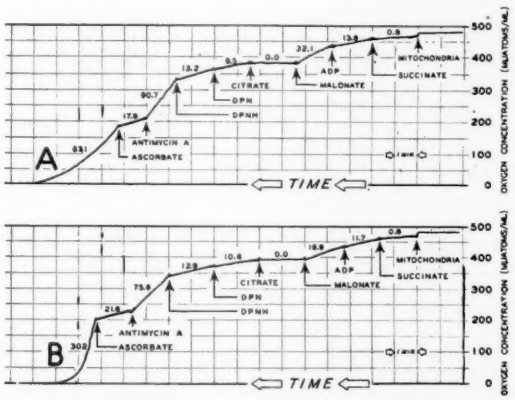

FIG. 1. Respiration curves at 26° C of mitochondria from normal and crown-gall tissue cultures of tomato. Recordings with Varian recorder. Figures in mμatoms/min/ml. Total reaction mixture 2.2 ml. Recording of the reactions was from right to left as traced from the original records.

A: Respiration curve of normal tissue mitochondria. Mitochondria, 134 μg N in 0.28 ml; succinate, 2.8 μmoles/ml; ADP: 0.2 μmole/ml; malonate, 14 μmoles/ml; citrate, 2.8 μmoles/ml; DPN; 0.5 μmole/ml; DPNH: 0.7 μmole/ml; Antimycin A, 0.4 μg/ml; ascorbate, 2.8 μmoles/ml. B: Respiration curve of crown-gall mitochondria. Mitochondria, 146 μg N in 0.28 ml. All others same as above (A).

OXIDATION OF KREBS CYCLE ACIDS AND DPNH: Table I shows rates of oxidation of DPNH, ascorbic acid, and the principal acids of the Krebs cycle by mitochondria from normal and crown-gall tissue cultures. These figures are based on several measurements for which a single substrate was tested to avoid the effects of other substrates, inhibitors, or change in oxygen tension which might be encountered in an experiment employing multiple compounds as in figure 1. Mitochondria from normal cultures oxidized the Krebs cycle acids tested more rapidly than mitochondria from crown-gall tissue cultures. With both normal and crown-gall particles the rates of oxidation of citrate, α-ketoglutarate, malate, and glutamate varied from preparation to preparation, but usually fell in the range of 0.4 to 0.8, when the rate of succinate oxidation was set to 1.0. DPNH was oxidized rapidly (about 7 times faster than succinate) both by normal and crown-gall mitochondria.

Mitochondria from both normal and crown-gall tissue cultures responded similarly to the cofactors and inhibitors tested (figs 1 & 2). Adding DPN enhanced oxidation of citrate, malate, α-ketoglutarate and glutamate 1.4- to 2-fold depending upon the preparation. The oxidation of DPNH was not affected by adding DPN or cytochrome c (fig 2, B), although the latter enhanced the oxidation rate about twofold when mannitol was omitted from the reaction mixture. Cytochrome c added to sucrose preparations of normal and crown-gall mitochondria increased the rate of oxidation of all the Krebs cycle acids tested by about 10 %. The oxidations of DPNH and of the Krebs cycle acids tested were inhibited about 90 % by Antimycin A (0.4 μg/ml) and cyanide (0.1 μmole/ml).

TABLE I

OXIDATION OF VARIOUS SUBSTRATES BY MITOCHONDRIA FROM NORMAL AND CROWN-GALL TISSUE CULTURES OF TOMATO

SUBSTRATE	NORMAL TISSUE		CROWN-GALL	
	O_2 UPTAKE $M\mu$ ATOMS/ MIN/MG N	RELATIVE ACTIVITY*	O_2 UPTAKE $M\mu$ ATOMS/ MIN/MG N	RELATIVE ACTIVITY*
Succinate	180	1.0	154	1.0
Citrate**	142	0.79	125	0.81
α-Ketoglutarate**	110	0.61	94	0.61
Malate**	85	0.47	75	0.49
Glutamate**	125	0.69	117	0.76
DPNH	1,250	6.9	1,090	7.1
Ascorbate	1,560	8.7	6,940	45.0

* Relative activity = Oxidation rate of substrate/ Oxidation rate of succinate.

** In the presence of DPN.

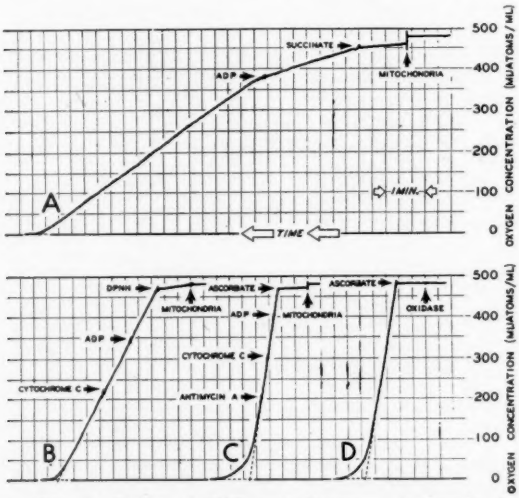

FIG. 2. Effect of ADP, cytochrome c, Antimycin A, and oxygen concentration on oxidation of succinate, DPNH, and ascorbate. Recordings with Brown recorder. Time scale for B, C, and D same as for A. Figures in mμatom/min/ml. Others same as in figure 1.

A: Oxidation of succinate by normal tissue mitochondria. B: Oxidation of DPNH by normal tissue mitochondria. C: Oxidation of ascorbate by crown-gall mitochondria. D: Oxidation of ascorbate by ascorbic acid oxidase extracted from cell debris fraction of crown-gall tissue cultures.

OXIDATION OF ASCORBIC ACID: Mitochondria from normal and crown-gall tomato tissue cultures oxidized ascorbate very rapidly; nine times faster than succinate by normal tissue mitochondria and 45 times faster by crown-gall mitochondria (table I). In contrast to the oxidation of Krebs cycle acids, ascorbic acid was oxidized by mitochondria from crown gall 4.5 times faster than by mitochondria from normal tissue. The mitochondrial oxidation of ascorbate was neither enhanced by DPN, ADP, or cytochrome c, nor inhibited by Antimycin A (fig 2,C). The addition of cyanide inhibited ascorbate oxidation. The minimum concentration of cyanide to cause maximum inhibition of ascorbate oxidation was 1.1 μmoles/ml, a concentration about ten times that required for maximum inhibition of DPNH oxidation (fig 3,A & B). Since the cell debris fraction of the tomato tissue cultures contained high ascorbic acid oxidase activity, ascorbic acid oxidase was extracted from this fraction. The sensitivity of mitochondrial oxidase to cyanide was similar to that of extracted ascorbic acid oxidase (fig 3,B & C).

The rate of ascorbic oxidation by the mitochondrial preparations decreased as the partial pressure of oxygen in the reaction mixture decreased (fig 2,C). Extracted ascorbic acid oxidase reacted in the same fashion (fig 2,D). This indicates a low affinity of the catalyst for oxygen. This contrasted with the oxidation of succinate or DPNH, in which the rate of oxidation was independent of the oxygen tension (fig 2,A & B).

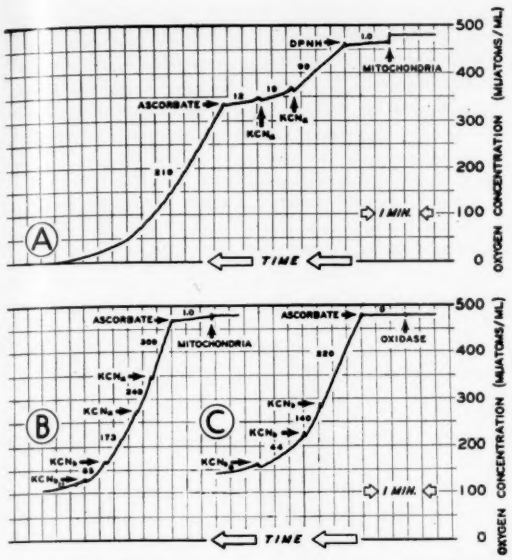

FIG. 3. Effect of cyanide on oxidation of DPNH and ascorbate by tomato tissue culture mitochondria and purified ascorbic acid oxidase. Recordings with Brown recorder. Figures in mμatoms/min/ml. KCN_a: KCN, 0.06 μmole/ml; KCN_b: KCN, 0.48 μmole/ml. Others same as in figure 1.

A: Oxidation of DPNH and ascorbate by crown-gall mitochondria. B: Oxidation of ascorbate by crown-gall mitochondria. C: Oxidation of ascorbate by ascorbic acid oxidase extracted from cell debris fraction of crown-gall tissue cultures.

EFFECT OF PHOSPHATE ACCEPTOR: Adding ADP or a mixture of ATP, glucose, and hexokinase increased the rate of oxidation of the substrates listed in table I except for DPNH (fig 2,B) and ascorbate (fig 2,C). With normal tissue particles, the increases were 1.6- to 2.5-fold (respiratory control ratios) for succinate (fig 1, fig 2,A), and 1.3- to 2-fold for the other substrates. In two experiments, 3.3- and 3.6-fold increases were observed for succinate oxidation. In comparison with the above, crown-gall particles gave lower respiratory control ratios; less than two for succinate oxidation.

The acceleration of respiration by ADP continued until all the oxygen in the reaction mixture was consumed, and there was no cessation of the enhanced respiration after the added ADP was supposedly exhausted by phosphorylation.

OXIDATIVE PHOSPHORYLATION: Table II shows phosphorylating efficiencies of mitochondrial preparations, respectively, from normal and crown-gall tissue cultures supplied succinate and citrate as substrates. With each substrate, particles from crown-gall gave considerably lower P/O ratios than did particles from normal tissue.

The P/O ratio in DPNH oxidation was less than half that obtained in succinate oxidation. No appreciable phosphorylation was observed to be coupled with oxidation of ascorbate.

DISCUSSION

In biochemical work on plant mitochondria, fast growing tissues from seedlings generally have been employed as the source of the particles (7). In the present study mitochondria from tomato cells cultivated in vitro were sufficiently active to examine respiratory and phosphorylative properties with the aid of an oxygen electrode apparatus.

A comparison of oxidation rates of Krebs cycle acids indicated that mitochondria from crown-gall tissue cultures had lower activity than mitochondria from normal tissue cultures. Crown-gall mitochondria also were lower in phosphorylating efficiency; the P/O ratio of crown-gall mitochondria was about 0.6 that of normal tissue particles. The lower oxidative and phosphorylative activities of crown-gall mitochondria contrast with the active growth of the tissue. This raises possibilities that A, crown-gall tissue cultures more efficiently utilize the energy produced than do normal tissue cultures, or B, crown-gall tissue cultures obtain energy other than through the energy-yielding system coupled with the oxidation of the Krebs cycle substrates.

The increase in respiration rate after ADP or phosphate acceptor (respiratory control) indicated tight coupling of the respiratory system to the phosphorylating system. This enhancing effect of a phosphate acceptor decreased with aging of mitochondria, and its decrease preceded a decrease in P/O ratio (11). Thus, the ability to exhibit respiratory control was a good indication of the quality of a mitochondrial preparation. The respiratory control has been shown with liver and kidney mitochondria (4- to 10-fold increase) (6, 11, 19), and to a lesser extent with particles isolated from heart muscle (4, 26, 27), ascites tumor cells (5), and certain plant species (7, 20). In succinate oxidation by mitochondria from normal tomato tissue cultures, the control ratio easily exceeded two; a value of 3.6 was obtained in one case. With crown-gall particles, on the other hand, the ratio rarely exceeded two.

TABLE II

COMPARISON OF PHOSPHORYLATING EFFICIENCIES OF MITOCHONDRIA FROM NORMAL AND CROWN-GALL TISSUE CULTURES OF TOMATO

EXP. NO.	SUBSTRATE	P/O RATIO	
		NORMAL TISSUE	CROWN-GALL
I	Succinate	1.0	0.66
	Citrate	1.1	0.67
II	Succinate	0.93	0.46
	Citrate	1.0	0.65

With rat liver mitochondria (6, 11) the ADP-enhanced respiration returned to the original rate when the added ADP was phosphorylated. Bonner and Ito (1) reported deceleration of respiratory rate with cauliflower mitochondria. This respiratory deceleration was not observed in the present experiments.

The rapid oxidation of ascorbate by mitochondria from normal and crown-gall tissue cultures deserves attention. In contrast to the Krebs cycle acids tested, ascorbate was oxidized more rapidly by mitochondria from crown-gall tissue cultures than by those from normal tissue cultures. The oxidation of ascorbate differed from the oxidation of the Krebs cycle acids or DPNH in A, insensitivity to Antimycin A, B, higher resistance to cyanide, and C, low affinity for oxygen (34). Thus, ascorbic acid oxidase seemed involved in this oxidation. In fact, purified ascorbic acid oxidase behaved similarly in a comparative experiment. Ascorbic acid oxidase has long been considered a soluble enzyme and has been highly purified from solution; however, recently at least some of its activity has been found to be associated with the cell surface (14, 22), and with mitochondria (21, 37). Since the cell debris fraction oxidized ascorbic acid rapidly, the mitochondrial oxidation of ascorbate from contaminating cell debris deserved consideration. However, the observations throughout the experiments supported the view that the mitochondria per se carried ascorbic acid oxidase.

Mitochondria from both normal and crown-gall tissue cultures oxidized DNPH rapidly (7 times as fast as succinate). Rapid oxidation of DPNH also has been reported with mitochondria from skunk cabbage (8), from lupine (15), and from many other plants (28). Spectrophotometric studies on DPNH oxidase, DPNH-cytochrome c reductase, cytochrome c oxidase, and diaphorase activities in mitochondria from normal and crown-gall tissue cultures are discussed in a forthcoming paper (32).

It may be concluded that the respiratory mechanisms of mitochondria from normal and crown-gall tissue cultures are quite similar. The only differences noted in their oxidative and phosphorylative activities were of a quantitative rather than a qualitative nature.

Summary

Oxidative and phosphorylative activities of mitochondria from normal and crown-gall tissue cultures of tomato were studied with an oxygen electrode. Mitochondria from crown-gall tissue cultures oxidized reduced diphosphopyridine nucleotide (DPNH), glutamate, succinate, citrate, malate, and α-ketoglutarate more slowly (about 0.9 times), but ascorbic acid more rapidly (4.5 times) than mitochondria from normal tissue cultures.

The phosphorylating efficiency of crown-gall mitochondria (P/O ratio, about 0.6 with succinate) was lower than that of normal tissue mitochondria (P/O ratio, about 1.0 with succinate). Adding a phosphate acceptor (adenosine diphosphate or glucose-hexokinase-adenosine triphosphate mixture) increased the rates of oxidation of the Krebs cycle acids tested, but not of DPNH and ascorbic acid. In succinate oxidation by normal tissue mitochondria, the enhancing effect by the phosphate acceptor was generally more than twofold (respiratory control ratio). With crown-gall mitochondria, on the other hand, the ratio rarely exceeded two.

The oxidation of ascorbic acid by the mitochondria from normal and crown-gall tissue cultures was compared to the oxidation by an ascorbic acid oxidase preparation; all the evidence obtained indicated that the mitochondria from both tissue cultures contained ascorbic acid oxidase.

Mitochondria from both normal and crown-gall tissue cultures responded similarly to cofactors (diphosphopyridine nucleotide, cytochrome c) and inhibitors (cyanide, Antimycin A).

In conclusion, the mitochondria from normal and crown-gall tissue cultures differed in their oxidative and phosphorylative activities in rather small quantitative rather than qualitative characteristics. Furthermore, mitochondria isolated from tomato tissue cultures, except for ascorbic acid oxidase activity, were similar in their respiratory properties to those from intact higher plants and animals.

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TRANSLOCATION OF CITRULLINE IN ALNUS GLUTINOSA [1]

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Since citrulline was first discovered as a constituent of the watermelon by Wada (10), it has been found in many other plants (1, 8, 9, 11). In the alder, this amino acid is the predominant nitrogenous constituent of all organs and accounts for a considerable fraction of the non-protein nitrogen (5, 8). Because of this Miettinen and Virtanen suggested that citrulline may be concerned with the translocation of nitrogen. The detection of citrulline in the bleeding sap of alder by Wolffgang and Mothes (11), and in xylem sap by Bollard (1) supported this contention. The evidence suggests that citrulline is travelling through the stem, but it was thought desirable to demonstrate this in a more direct manner with an isotopic tracer —a method potentially capable of yielding much more precise and detailed information about the movement of this and other amino acids. As tracer, N^{15} has obvious advantages in this context, but in practice C^{14} was preferred on account of the greater convenience and much higher sensitivity of the methods for the assay of the carbon isotope. Earlier studies (5) had indicated that the synthesis of citrulline in alder probably involves fixation of carbon dioxide, and this suggested a convenient way of labeling citrulline with C^{14} in the root of the plant and thus of following its subsequent distribution. It has now been demonstrated that roots of alder do readily fix carbon dioxide in citrulline, and this observation has enabled us to demonstrate the movement of citrulline, formed in the root system, to shoot and leaves.

EXPERIMENTAL METHODS

A small plant of *Alnus glutinosa* (L.) Gaertn., aged 14 months and 20 cm in height, was placed with its roots in Crone's solution (2) contained in a small flask. A solution of radioactive bicarbonate (40 μc, 3.36 mg $NaHCO_3$) was added to the medium and the plant was left in sunlight for 4 hours. At the end of this period, organs were removed and ground in a mortar with acid-washed sand together with nine times their weight of ice-cold 0.1 N HCl. The resulting suspension was centrifuged at 0° C and the supernatant solution lyophilized. The dried material was taken up in water and suitable portions of this solution were taken for determination of total N (3), total radioactivity, and for chromatographic analysis. The insoluble residue from the extraction was dried, a sample was heated with 6 N HCl at 115° C for 15 hours, and the amino acids thus liberated were separated by chromatography on paper.

CHROMATOGRAPHIC ANALYSIS. A sample of each extract was freed from interfering ions (5) and subjected to two-way chromatography with butanol-acetic acid-water (4:1:5) as the first solvent and water-saturated phenol, in the presence of ammonia, as the second. After spraying the chromatograms with ninhydrin and zinc sulphate, radioautographs were taken on Kodak Industrex D film with an exposure of 21 days. This procedure was repeated with a sample of the extract after hydrolysis with 6 N HCl at 115° C for 15 hours.

DETERMINING SPECIFIC ACTIVITIES OF AMINO ACIDS. From a third sample of extract, the amino acids were isolated as their dinitrophenyl (DNP) derivatives by the paper chromatographic method of Koch and Weidel (4). The amount of each DNP amino acid was determined spectrophotometrically. The solution was then acidified, the DNP-amino acid extracted with ethyl acetate, and a suitable portion of extract was evaporated on a planchet for determination of its radioactivity. Under the conditions used by Koch and Weidel (4), DNP-citrulline was separated from the DNP derivatives of all other commonly occurring amino acids except glutamine. When both were present, the material extracted from the citrulline spot was heated with 1.0 N HCl for 2 hours at 100° C. This converted DNP-glutamine to DNP-glutamic acid which was readily separated from DNP-citrulline by chromatography on paper with 1.5 M phosphate buffer (7).

This method was found satisfactory for determining specific activity, where quantitative isolation is unnecessary, but erratic recovery of DNP-amino acids from alder extracts made it unsuitable for accurate determination of amino acids in these extracts.

RESULTS

FREE AMINO ACIDS OF ALDER TISSUES. This matter was previously investigated by Miettinen and Virtanen (8). In contrast to the findings of these authors, we have found glutamine to be present in the roots of alder though not in other organs. With the solvent systems commonly used for paper chromatography of amino acids, the spot of glutamine tends to overlap that of citrulline, and this makes their independent detection by routine chromatographic examination difficult. The appearance of radioautographs, however, indicated that the material in the citrulline spot, from root extracts, was inhomogenous. The appropriate area from a duplicate chromatogram was therefore excised, the amino acids were extracted and the solution was heated with N HCl at 100° C for 2 hours. Paper chromatography now gave a more

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compact spot in the citrulline position, and a new radioactive spot appeared in the glutamic acid position. The conclusion that the contaminant was glutamine was confirmed by analysis of the DNP derivatives. The molar concentration of glutamine present in the root extracts was approximately the same as that of citrulline.

In other respects, our observations agreed with those of Miettinen and Virtanen (8). In the extracts from nodules, roots, and leaves, aspartic acid, glutamic acid, and citrulline were predominant, and the last always accounted for the greatest part of the nitrogen of the extract. Other amino acids—serine, threonine, alanine, valine, leucine (and/or isoleucine), and γ-aminobutyric acid—were present in much smaller amounts; their concentrations being below the concentrations at which they could be isolated as their DNP derivatives under conditions suitable for isolating the derivatives of aspartic acid, glutamic acid, and citrulline. In extracts of the stem, citrulline was accompanied only by much lower concentrations of aspartic and glutamic acids. The pattern obtained with xylem sap, extracted as described by Bollard (1), was similar; so similar indeed, as to suggest that most of the nitrogen in the acid extract was derived from the xylem. In contrast to the finding of Bollard (1), we were unable to detect glutamine in the xylem sap.

In an earlier paper (6), we reported the presence of hydroxyproline among the amino acids formed on hydrolysis of the insoluble protein fraction from Myrica nodules. We have now found this amino acid in hydrolysates of similar fractions from nodules and stem of alder. The amounts present were less than in Myrica nodules.

Distribution of C^{14}. This was first investigated by radioautography. The findings of this preliminary experiment were then confirmed and extended, on a second plant, using both radioautography and the quantitative methods described above. The radioactivities of the fractions examined are given in table I. The proportion of the total radioactivity accounted for by DNP-amino acids, extractable by ethyl acetate, varied from 10 % to 29 %. For leaves it was 10 % and this indicated that C^{14} was reaching the leaves in the form of compounds other than amino acids. The nature of these has not been investigated.

In the amino acid fraction, the greatest part of the radioactivity was always found in citrulline, and this amino acid always showed the highest specific activity. Radioautographs of chromatograms made from hydrolysed extracts showed that ornithine derived from the citrulline had a marked radioactivity; the fixed carbon was not restricted to the carbamyl group in the citrulline. Radioautographs prepared from untreated extracts showed a few radioactive spots apart from those of the amino acids mentioned in table I; but these were faint and they disappeared on hydrolysis. In nodule extracts there was a small amount of arginine and this was weakly radioactive; but its concentration was too low for determination of specific activity.

Discussion

The values for the specific activities of citrulline derived from various parts of the plant are quite compatible with the view that citrulline is synthesised in the root system and transported up the stem to the leaves and the shoot. Table I, however, shows that much more C^{14} found its way up the stem than was carried in the form of citrulline. It is therefore necessary to consider whether or not the radioactive citrulline in the leaf may have been synthesised from C^{14} carried there in some other form. It is possible to reject this conclusion on two counts. Glutamic acid was heavily labeled in the roots but, though this amino acid was found in the stem, its specific activity was very low and glutamic acid in the leaves showed no detectable activity. The simplest explanation for the differences between the two amino acids is that citrulline is readily transported from the roots whereas glutamic acid is not. This argument is strengthened by the observation that the ornithine derived from citrulline of both stem and leaves showed radioactivity.

Table I

Distribution of C^{14} in Free Amino Acids of Alder Organs

	Total activity of extract cpm/μatom N*	Total cpm/μatom N*	Activity of DNP-amino acids: Specific activities cpm/μmole			
			Citrulline	Glutamine	Glutamic acid	Aspartic acid
Root nodules	62.3	18.1	615	—**	219	288
Roots	52.1	5.88	2,060	557	488	158
Stem	47.3	11.8	425	—	43	0
Leaves	8.68	0.87	145	—	0	0

* Per μatom N in original extract.

** A dash - indicates that the amino acid was not detectable in the extract; O that the amino acid was present but showed no measurable radioactivity.

It appears most unlikely that, in the leaf, C^{14} could be incorporated into ornithine without appearing in glutamic acid.

When these experiments were begun, it was hoped that labeling the citrulline in the manner described would make possible a quantitative study of the translocation of this compound and also give some indication of its fate on arrival in the leaves. The present results show that the rates of incorporation of C^{14} into citrulline and translocation of the radioactive amino acid are too slow relative to the total movement of C^{14}, for this purpose. In longer experiments, required for the study of the fate of the translocated citrulline, the parallel translocation of C^{14} would make interpretation of the results difficult. Other methods of obtaining this information are therefore being investigated.

The occurrence of glutamine in alder roots is of interest, for it has been assumed that, with respect to functions which call for or give rise to a large reserve of the substance, citrulline replaces glutamine in the alder. Glutamine appears to be absent from other organs, and although Bollard (1) reported its presence in xylem sap of alder plants, we were unable to confirm this observation with xylem sap extracted from field alders. In this connection it is important to mention that our experimental plants were given combined nitrogen (nitrate), and it is possible that the occurrence of glutamine in the roots may be associated with the availability of combined nitrogen which could be directly absorbed by the roots. This possibility is under investigation.

Summary

Young alders absorbed $HC^{14}O_3^-$ from the root medium and incorporated its carbon into aspartic and glutamic acids, glutamine, and citrulline. After 4 hours, citrulline was the only amino acid to contain C^{14} in the leaves, although glutamic acid in the stem also showed a weak activity. Glutamine was found in the roots but it did not appear to undergo translocation. These results are compatible with the view that citrulline is the main vehicle for the translocation of nitrogen in the alder.

Acknowledgments

We wish to thank the Commissioners for the Exhibition of 1851 for the Senior Studentship held by one of us (I.C.G.), and I.A.N. Napier for technical assistance.

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LIGHT DOSAGE AND PHOTOTROPIC RESPONSES OF CORN AND OAT COLEOPTILES[1,2]

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For a number of years, it has been assumed that the Bunsen-Roscoe reciprocity law is valid for phototropic curvature (21, 30). This law states that if light intensity and time of exposure are varied in such a way that the product of the two remains constant, the photochemical effect of the light should also remain constant (13). Thus it was thought that if plants were exposed to unilateral light, a uniform curvature response could be obtained, all other conditions being constant, provided only that the product of light intensity and exposure time remained the same. Indeed, Fröschel (19) and Blaauw (4) showed the reciprocity law to be valid over a wide range of intensities for Lepidium seedlings and Avena coleoptiles, respectively. However, their investigations were limited to the low dosages necessary to elicit the first measurable response. Similarly Fuller and Thuente (20) confirmed the law for a number of plants, again only for low dosages. Haig (23) subsequently confirmed Blaauw's results with Avena coleoptiles using unilateral "violet" light and low dosages. Numerous other examples are discussed by Du Buy and Nuernbergk (16).

Thus the reciprocity law is generally accepted for phototropism (21, 30) despite the fact that confirmation at high dosages has not been obtained. Indeed, Arisz (1) clearly demonstrated that the law is not valid for negative curvatures; he suggested that it is only of limited applicability, and Bremekamp (10) concluded that it was valid neither for negative curvature nor for "second positive" curvature (see below). There are references, furthermore, indicating that the law is not necessarily valid either if the stimulus is intermittent rather than continuous (14, 22) or if it is administered in anything but a strictly unilateral fashion (1, 10, 16, 28). These various exceptions will be considered in the discussion.

In 1934, Du Buy and Nuernbergk (15) suggested a phototropic dosage-response curve for Avena coleoptiles. From their results, Went and Thimann (33) plotted curvature response to unilateral light against the logarithm of the dosage of white light in meter-candle-seconds (MCS), and obtained the graph reproduced in figure 1. The three response peaks are usually designated first, second, and third positive curvature; and the valley between first and second positive curvature, which is actually negative, is designated first negative curvature. It should be pointed out that Went and Thimann's curve is actually a simplification of the data of Du Buy and Nuernbergk, since reaction times following the beginning of the light stimulus were not indicated, and were not the same for all dosages. Thus the absolute magnitude of curvature is less significant for the present consideration than the direction of curvature with respect to light dosage.

Preliminary experiments with corn coleoptiles (11) indicated that the reciprocity law is not valid over the complete range of light dosages indicated in the Du Buy and Nuernbergk curve (fig 1). Above a certain dosage (1,000 MCS) the law fails at high intensities and the magnitude of the curvature response decreases. As the dosage is progressively increased above this value, failure of the law becomes increasingly drastic. The present study will describe results of experiments investigating phototropic reciprocity relationships of Avena and Zea coleoptiles over a wide range of light dosages. The bearing of these results on the shape of the phototropic dosage-response curve will be considered, and experiments suggesting possible reasons for the failure of the reciprocity law at intermediate and high dosages will be discussed.

Zea coleoptiles were selected for the majority of the experiments described below since a certain amount of information was already available concerning their auxin relationships during phototropism (12). These coleoptiles are more suitable for auxin diffusion studies than those of Avena since they are larger in cross-sectional area, and consequently easier to manipulate. Phototropic auxin diffusion studies in addition to those mentioned above have been made and will be discussed in detail in another paper.

MATERIALS AND METHODS

Corn seeds (*Zea mays* L., var, Burpee Snowcross) were surface sterilized for 5 minutes in 2% commercial hypochlorite (Purex), rinsed for 2 hours in running deionized tap water, and then set embryo up on moistened filter paper in finger bowls. When the shoots were about one centimeter in length (72 hr

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after soaking), each seed was transferred to a plastic vial. The root extended into deionized tap water, the seed was just above the water, and the coleoptile passed through a small hole in the lid of the vial. The vials were assembled into rows of eight, on wooden racks. It was possible to rotate the vials so that the straightest vertical dimension of each coleoptile was in the plane of the rack, and at right angles to the unilateral light source. Due to the morphology of the corn seedling, this procedure placed the longest horizontal axis of each coleoptile in a direct line with the light source, and therefore, likewise, the plane of the two vascular strands. The coleoptiles were exposed to unilateral light when they were between 2.0 and 2.5 cm in length, usually about 90 hours after the initial soaking. Following the light exposure, each vial was rotated 90° around its vertical axis, so that the curvature of each plant developed in the plane of the row. Early experiments indicated that curvature was at a maximum 2 hours following the beginning of the light exposure. Thus at this time shadowgraphs were made of each row on glossy photographic paper, and permanent records of curvatures obtained.

These were measured with a goniometer such as is normally used to measure Avena curvature (33). In the data presented below, each curvature value represents the average of eight plants. Since the corn coleoptile is an unfortunately variable organ both in size and phototropic sensitivity, and standard errors were usually high (min ± 0.57°, max ± 4.4°, avg ± 2.3°) despite rigorous selection of plants, each experiment was done on several occasions. Thus although single experiments are presented below, each is one of two, three, or sometimes four comparable tests.

One difficulty arose from the fact that the first internode of this variety of corn can be only partially suppressed by red light. Thus, at the time of exposure, each plant possessed a rather irregular first internode with its own phototropic sensitivity. It was therefore necessary to shield the plants during exposure so that no light reached either the base of the coleoptile or the internode below. However, all but approximately the lowest 2 mm of each coleoptile was exposed to light, rather than just the tip. Since at high light intensities it was possible that sufficient light scattering might occur to bring about a phototropic response from the lower and non-illuminated parts, the magnitude of coleoptile curvature was determined by measuring only that part of the coleoptile more than 2 mm above the node. Plants in which the node showed obvious curvature were discarded.

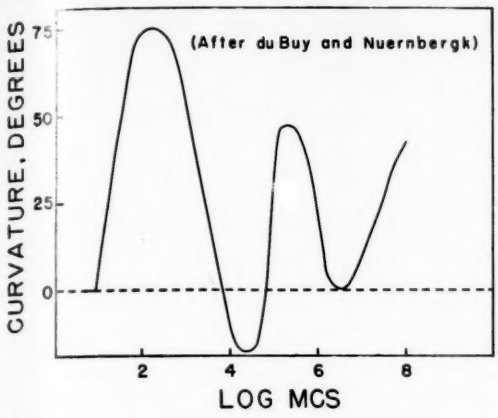
Victory oats (*Avena sativa* L. var. victory) were hulled and soaked in tap water for exactly 2 hours. They were then set embryo up in petri dishes on two layers of moist filter paper and exposed to 4 hours of red light to suppress the first internodes. Twenty-four to thirty hours after the initial soaking, the seeds were individually planted with the roots extending into 1% agar in test tubes. The tubes were assembled in rows of 10 or 11 in wooden racks, and each row of plants was exposed to unilateral light approximately 72 hours after soaking, when the coleoptiles were between 2.5 and 3.0 cm in length. The seeds, reduced first internodes, and lowermost millimeter of the coleoptiles were shielded from light as with corn. Following light exposure the tubes were rotated 90° as described above. Curvatures were allowed to develop for 100 minutes, at which time shadowgraphs were made. The curvatures were measured as with corn: each value shown below represents the average curvature of 10 or 11 plants. The Avena results shown are typical of two or more comparable experiments. Maximum standard errors never exceeded ± 2.9° (avg ± 1.74°).

A standard 40 or 100 w incandescent bulb was used as a light source, and intensity, determined with a Weston foot-candle meter, was varied by changing the distance between the plants and the light source. In experiments involving flashing light, light from the incandescent source was passed through a slit, through a rotating disc with a sector of measured size removed, through another slit, and then through a lens which focused the image of the disc sector on the plane of the plants being exposed. The speed of rotation of the disc was controlled with a variable-speed motor, and the revolutions per minute were determined by means of a stroboscope (Strobotac, General Radio Corp). Relative lengths of the light and dark periods could be controlled by varying the size of the disc sector removed, and the length of the light flash could then be adjusted by varying the speed of rotation of the disc.

Following the initial soaking, both corn and oat seeds were allowed to germinate and grow in a room maintained at 25° C and 85% relative humidity. With the exception of the initial red irradiation of the oats, both species were grown in the dark save during experimental manipulations during which dim red light was used.

Experimental Results

Early attempts were made to obtain a phototropic dosage-response curve for corn coleoptiles, for comparison with that mentioned above for Avena (15). It was hoped that a knowledge of the dosage-response relationships of corn coleoptiles might aid in interpreting the various auxin diffusion experiments which had been done. A typical curve for corn is shown in figure 2. In this series of experiments, for practical reasons of time limitation, total dosage was increased as far as possible by increasing light intensity, and only relatively short light exposures were used. Thus exposure times were less than 5 minutes in all cases except those represented by the last solid circle to the left and the last two open circles to the left. Comparison of figure 2 with figure 1 indicates that the two curves are roughly the same shape in the range of dosages common to the two, although the first maximum and minimum for corn coleoptiles occur at slightly higher dosages than those for Avena, and



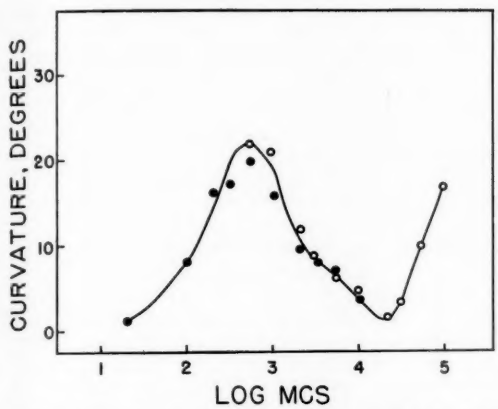


FIG. 1 (*Top*). Relationship between unilateral light dosage (log MCS) and phototropic curvature of Avena coleoptiles. After Du Buy and Nuernbergk (15) and Went and Thimann (33).

FIG. 2 (*Bottom*). Relationship between unilateral light dosage (log MCS) and phototropic curvature of corn coleoptiles. All exposure times less than 5 minutes except where noted in text.

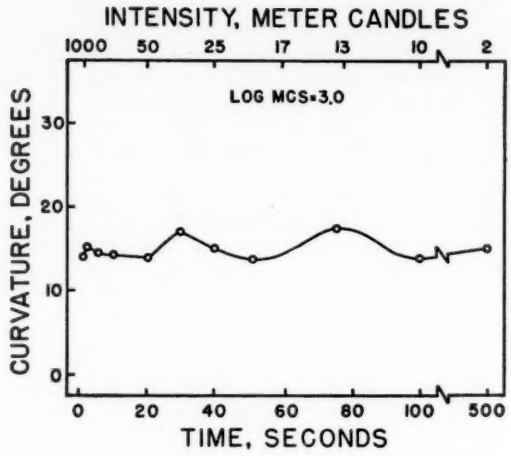
corn showed no true negative curvature. Figure 2 is derived from two separate experiments which overlapped in the middle range of dosages (solid and open circles). However, attempts to extend the curve to higher dosages, using a brighter light source than that described above, with a water filter to prevent heat damage and still shorter exposure times, resulted in little or no curvature, even in dosage ranges in which substantial curvatures had previously been obtained. Apparently the manner in which the light was administered had a marked effect on the magnitude of the phototropic response at the higher dosages.

PHOTOTROPIC RECIPROCITY RELATIONSHIPS OF CORN COLEOPTILES: Since the experiments described above indicated that the reciprocity law was not necessarily valid for corn coleoptiles exposed to relatively high dosages of unilateral light, a series of experiments was designed to test the reciprocity law for a wide range of dosages. For each dosage tested, light intensity and time were varied reciprocally, and the results of some such experiments are shown in figures 3 to 8. These figures represent successive logarithmic increments of 0.5 for the dosages, covering a range from 1,000 to 316,000 MCS inclusive. Additional experiments covering intermediate logarithmic increments of 0.25 supported the general pattern shown. An examination of figure 3 indicates that for a dosage of 1,000 MCS, the reciprocity law is valid for corn, at least between intensities of 2 and 1,000 meter-candles. However, figures 4 to 8 indicate that as the dosage is progressively increased, failure of the reciprocity law becomes increasingly evident. The amount of time during which the dosage must be administered to obtain maximum response and validity of the reciprocity law increased from approximately one minute (log MCS = 3.5) to more than 35 minutes (log MCS = 5.5).

Figures 7 and 8 indicate another feature of considerable interest. As the light intensity in these two cases is progressively increased, the curvature response gradually decreases to zero. However, continued increase of the light intensity above 1,000 meter-candles produces a significant amount of positive curvature, more pronounced at the higher dosage (log MCS = 5.5). Auxin distribution studies to be considered elsewhere suggest that the positive curvatures to the left and right of the minimum may be mediated by different mechanisms.

Figure 9 illustrates the relationship between dosage and the approximate maximum limiting intensity above which the reciprocity law fails. It will be recalled (fig 3) that at a dosage of 1,000 MCS such failure does not occur, at least at intensities up to 1,000 meter-candles. Furthermore, the literature cited in the introduction indicates that it does not occur for lower dosages at even higher intensities than those used in the present study. As the dosage is progressively increased however, a limiting intensity is suddenly imposed, and decreases rapidly to a minimum of about 30 meter-candles when the dosage is 10,000 MCS. As the dosage continues to increase, the limiting intensity then begins to increase. This curve suggests that at dosages of 10,000 MCS or less, one mechanism, mediated by one pigment system, may be operative, while above this dosage, a second and somewhat less sensitive mechanism, mediated by a pigment system both less sensitive and perhaps with a different pattern of failure, may become of increasing importance. This possibility will be considered in the discussion.

PHOTOTROPIC DOSAGE-RESPONSE CURVE FOR CORN COLEOPTILES: A study of figures 3 to 8 indicates that for dosages between 1,000 and 10,000 MCS, optimum conditions of light intensity and time yield average curvatures of approximately 20° or less.


INTENSITY, METER CANDLES
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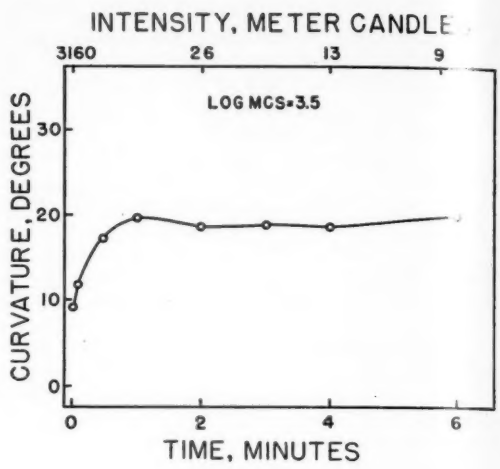
LOG MCS=3.0
CURVATURE, DEGREES
0 10 20 30
0 20 40 60 80 100 500
TIME, SECONDS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CURVATURE, DEGREES
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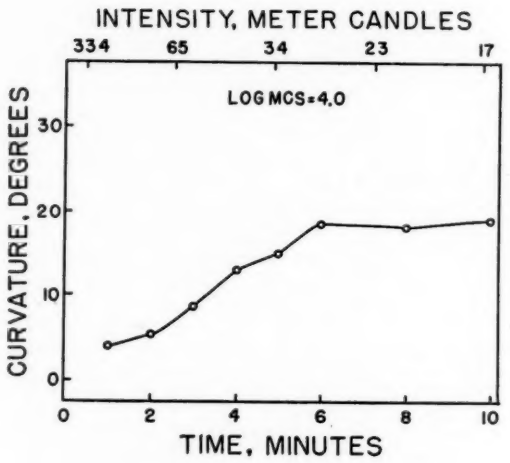
0 2 4 6
TIME, MINUTES


INTENSITY, METER CANDLES
334 65 34 23 17
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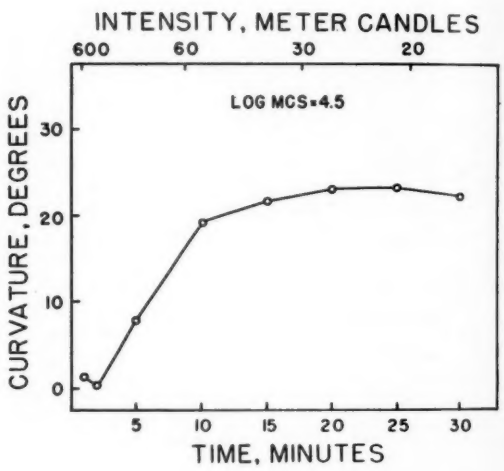
CURVATURE, DEGREES
0 10 20 30
0 2 4 6 8 10
TIME, MINUTES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CURVATURE, DEGREES
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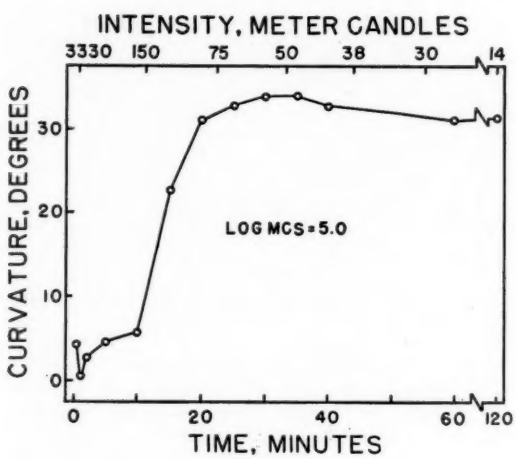
5 10 15 20 25 30
TIME, MINUTES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CURVATURE, DEGREES
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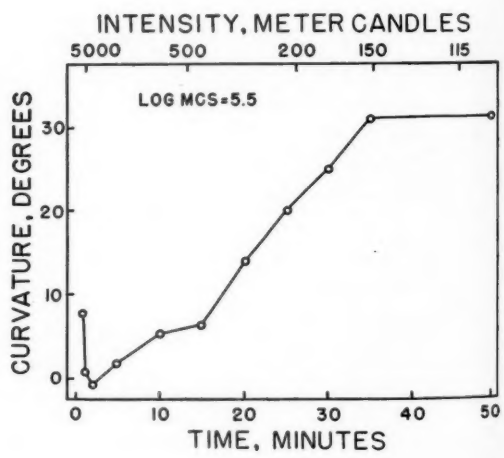
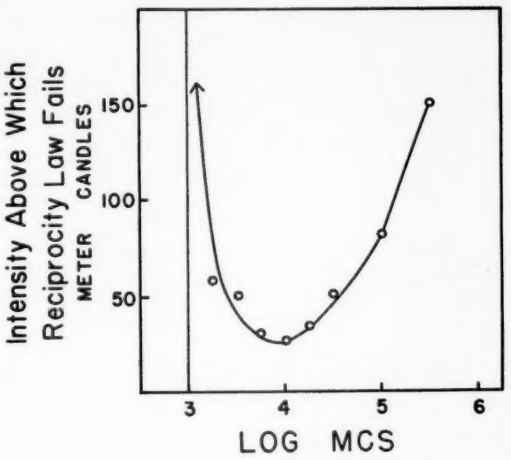
0 20 40 60 120
TIME, MINUTES


INTENSITY, METER CANDLES
5000 500 200 150 115
LOG MCS=5.5
CURVATURE, DEGREES
0 10 20 30
0 10 20 30 40 50
TIME, MINUTES

However, at higher dosages, the amount of curvature obtained under optimum conditions is considerably higher, either approaching or slightly exceeding 30°. These results suggest that a dosage-response curve obtained under conditions of light intensity and time suitable for maximum curvature might be quite different from that shown in figure 2, obtained using short and relatively high intensity exposures. Figure 10 illustrates a typical dosage-response curve obtained when care is taken that each dosage is administered under conditions in which the reciprocity law is valid. The curve is step-shaped, with a first and second increment corresponding to the first and second positive curvatures of the earlier graphs (figs 1 and 2).

It has long been known that the tip of the Avena coleoptile is approximately 1,000 times as sensitive to unilateral light as the more basal regions (26). Frequent distinction has been made between "tip curvature" and "base curvature" to distinguish between the phototropic responses of these two regions, and the two responses are discussed in detail in a review by Went (32). The experiments of Boysen-Jensen (6) and Boysen-Jensen and Nielsen (7) clearly show that direct contact between lighted and shaded sides of an Avena coleoptile is necessary in order to obtain maximum phototropic curvature. These workers inhibited such curvature by the inserting of small fragments of platinum foil through the middle of the coleoptile tip perpendicular to the plane of the light beam. Their results are frequently used to support the concept of light-induced lateral migration of auxin as an important factor in tip curvature (8, 21, 32).

Accordingly the experiment illustrated in figure 11 was done. Coleoptiles a and d represent control rows exposed to optimum first and second increment dosages, respectively. Coleoptiles b and e represent plants with their tips split parallel to the plane of illumination. Their curvatures in response to unilateral light were reduced somewhat in comparison to the controls, but were not eliminated. Coleoptiles c and f represent plants with their tips split perpedicular to the plane of illumination. In cases b, c, e, and f, the splits were approximately two millimeters deep, and the halves of the split tips were kept physically separated by means of small fragments of cover glass. The results shown by the left-hand coleoptiles indicate that in the case of first increment curvature alone (log MCS = 3.0) the presence of a physical barrier in the tip at right angles to the light source will completely eliminate any curvature response. Apparently first increment curvature is tip curvature alone, and



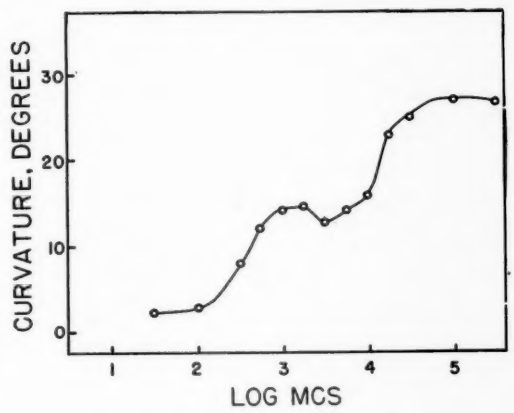


FIG. 9 (*Top*). Relationship between light intensity above which the reciprocity law fails (ordinate) and log dosage (abscissa). Details in text.

FIG. 10 (*Bottom*). Relationship between unilateral light dosage (log MCS) and phototropic curvature of corn coleoptiles when all dosages are administered at intensities within the range of validity of the reciprocity law.

FIG. 3 (*Top left*). Curvature response of corn coleoptiles to 1,000 MCS unilateral light with reciprocal variation of intensity and exposure time.

FIG. 4 (*Top right*). Same as figure 3, but with dosage of 3,160 MCS.

FIG. 5 (*Center left*). Same as figure 3, but with dosage of 10,000 MCS.

FIG. 6 (*Center right*). Same as figure 3, but with dosage of 31,600 MCS.

FIG. 7 (*Bottom left*). Same as figure 3, but with dosage of 100,000 MCS.

FIG. 8 (*Bottom right*). Same as figure 3, but with dosage of 316,000 MCS.

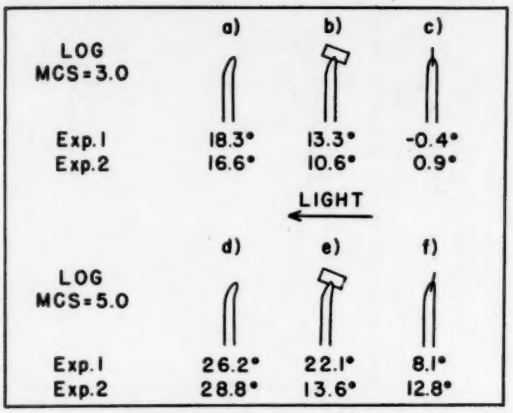
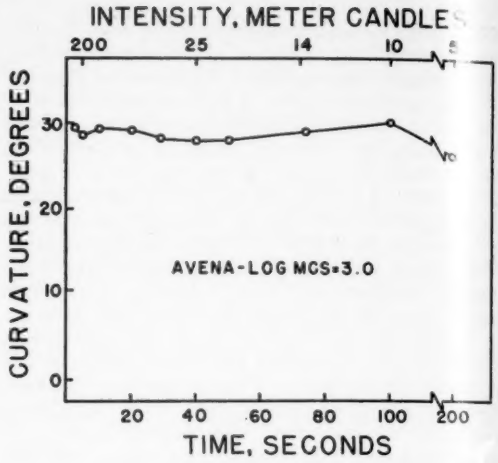
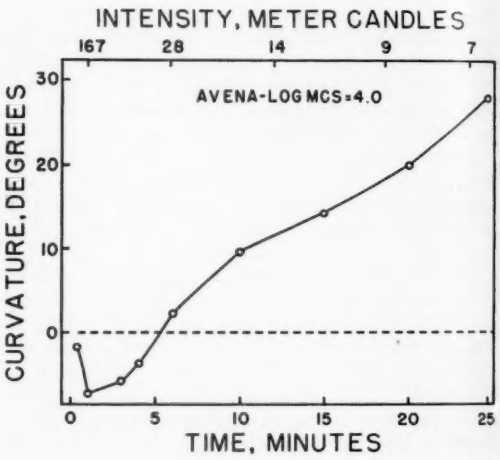


FIG. 11. Curvatures of corn coleoptiles in response to first and second increment dosages of unilateral light. Dosages administered within range of validity of reciprocity law. (a and d) Coleoptiles intact. (b and e) Coleoptile tips split parallel to light source, fragments of cover glass inserted in splits. (c and f) Coleoptile tips split perpendicular to light source, fragments of cover glass inserted in splits. Details in text.

mediated by lateral migration of some substance, probably auxin or a precursor (12). However, in the second increment curvature (log MCS = 5.0) the physical barrier reduces but does not eliminate the curvature response. It seems likely that second increment curvature is the result both of tip curvature, presumably mediated by lateral migration of auxin or a precursor in the coleoptile apex, and base curvature, which is independent of tip curvature. This hypothesis is supported by the fact that in both experiments shown, tip curvature alone, a, plus second increment curvature obtained with the barrier present, f, equals total second increment curvature, d. Auxin diffusion studies to be presented elsewhere indicate that base curvature is probably largely independent of an auxin differential in corn coleoptiles, and is most likely a light-growth reaction of the type referred to by several earlier workers (*27, 29, 32*). Such a reaction involves a lessening in sensitivity to auxin of the cells on the lighted side of the coleoptile in comparison to those on the shaded side, with a resulting curvature developing throughout the growing region.





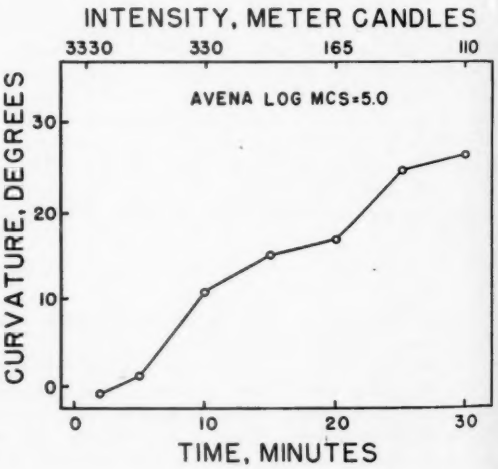


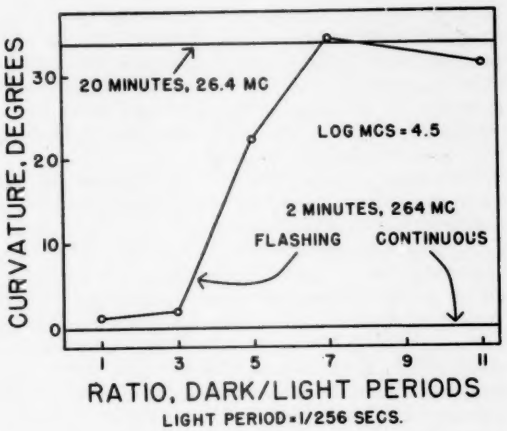
FIG. 12 (*Top*). Curvature responses of Avena coleoptiles to 1,000 MCS unilateral light with reciprocal variation of intensity and exposure time.

FIG. 13 (*Center*). Same as figure 12, but with dosage of 10,000 MCS.

FIG. 14 (*Bottom*). Same as figure 12, but with dosage of 100,000 MCS.

PHOTOTROPIC RECIPROCITY RELATIONSHIPS OF AVENA COLEOPTILES: A series of experiments testing the validity of the reciprocity law at different light dosages was carried out using Avena coleoptiles, to determine whether or not Avena is in any way comparable to corn. Figures 12 to 14 show representative results. They resemble those obtained with corn, but certain obvious differences should be noted. As with corn coleoptiles, a dosage of 1,000 MCS may be administered over a wide range of intensities and times without affecting the magnitude of the curvature response (fig 12). Thus the reciprocity relationships described by Blaauw (4) for dosages necessary to elicit first measurable response are also valid at this dosage. Furthermore, as the dosage is increased, the reciprocity law fails, just as for corn, and increasing dosage brings about progressively more drastic failure. Inspection of figures 13 and 14 shows, however, that for the ranges tested, the law was never valid. Both of these curves have been extended considerably to the right, and no consistent leveling, to be expected when the reciprocity law is valid, has been found. Thus the photosensitive system or systems in Avena presumably fail at far lower intensities than do those in corn. The overlapping of phototropic induction with curvature response in time however, is a far more serious problem with Avena than with Zea, since Avena coleoptiles begin to show curvature sooner than corn coleoptiles. Thus, with long light exposures simultaneous phototropic and geotropic induction undoubtedly occurs toward the end of the light exposure, and the resulting curvature is in no sense a simple function of the light stimulus. It is hoped that phototropic induction at low temperatures, in the absence of growth, followed by curvature development at 25° C on a clinostat will provide a means of separation of induction from response, and provide a more accurate study of Avena reciprocity relationships than that above. The low temperature technique has been successfully used by Brauner and Hager (9) for studying the geotropic response of Helianthus hypocotyls.

Figure 13 illustrates another difference between the response of Avena and that of corn. At a dosage of 10,000 MCS (log MCS = 4.0), increase in light intensity and reciprocal decrease in exposure time result in progressively lower response, until an intensity of 30 meter-candles produces no response at all. This pattern is comparable to that of corn. However, further increase in light intensity results in true negative curvature. This experiment was repeated on five separate occasions, always with comparable results. It would seem reasonable that the phototropically sensitive system eventually fails completely with increasing light intensity, but that the optical properties of the Avena coleoptile are such that under certain conditions enough light passes through the tip that the photosensitive system on the shaded side becomes activated, and the ultimate response is the reverse of normal. When light intensity is further increased, one would expect the system on the shaded side to fail also, and the curvature response to return to zero, as it is observed to do. In order to have such a system produce negative curvature, the coleoptile would have to absorb just the correct amount of light such that when complete failure occurred on the lighted side, the shaded side would be activated. The absence of true negative curvature in corn coleoptiles suggests that these organs do not possess these precise



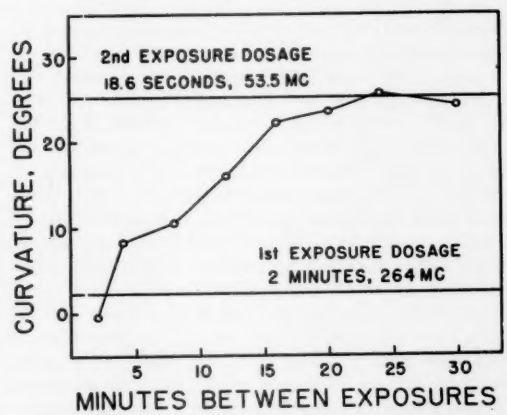


FIG. 15 (*Top*). Effect of flashing light on the phototropic response of corn coleoptiles. All dosages 31,600 MCS. Top line: maximum curvature obtainable under conditions of complete validity of reciprocity law. Bottom line: response under conditions of complete failure of reciprocity law. Experimental curve: same conditions of intensity and time as bottom line, but light administered in flashes. Details in text.

FIG. 16 (*Bottom*). Effect of dosage of 31,600 MCS (1st dosage) administered under conditions of complete failure of reciprocity law, on subsequent sensitivity of corn coleoptiles to normally effective first increment dosage (2nd dosage). Bottom line: normal response to first dosage. Top line: normal response to second dosage administered without first. Details in text.

relationships of pigment system failure and light absorption by the tips.

Response Failure of Corn at High Light Intensities: There are several reasons why the phototropic system might be expected to fail at high light intensities. One of these is that a large amount of light scattering might obscure the light differential between lighted and shaded sides. A second is that the phototropic response might be limited by a thermochemical dark reaction as was observed to be the case with photosynthesis (17, 18). A third is that sustained high intensity light might result in receptor pigment inactivation by photo-oxidation or some other reaction. A fourth possibility, to be considered in the discussion, is that light in some way directly inhibits the lateral translocation system.

The first possibility, based on a disappearance of the light differential, may be readily eliminated by the results of flashing light experiments similar to those used for photosynthesis (17, 18). Figure 15 shows the results of a typical experiment. In all cases shown in this figure, the total dosage was 31,600 MCS (log MCS = 4.5). The upper line represents curvature obtained from control plants under conditions of light intensity and exposure time within the range of validity of the reciprocity law. The lower line represents the behavior of plants under conditions of complete failure of response to the same dosage. The intensity has been increased tenfold and the time decreased to one-tenth that of the upper controls. The curve between the two lines represents the curvature response to the exact conditions of intensity and time represented by the lower control line, but with the light administered in flashes. Preliminary experiments had indicated that if one decreased the length of both light and dark periods simultaneously, and if they were both of equal length, a slight curvature would be obtained with light and dark periods of 0.004 seconds each. Figure 15 illustrates results obtained when the light period was maintained at this duration, and the dark period progressively increased. A curvature response was gradually recovered, and with a dark period of 0.027 seconds or above, maximum curvature was obtained. Obviously if obscuring the light differential between lighted and shaded sides by any means accounted for failure of the reciprocity law, administering the light intermittently should not affect the curvature response.

The possibilities of a limiting thermochemical reaction following pigment activation, or of inactivation of the receptor pigment or pigments, remain to be considered. The first of these seems unlikely, at least by itself. If pigment activation were occurring at a more rapid rate than could be accommodated by a limiting dark reaction, one would expect a less than optimal response, but hardly no response at all. The type of experiment illustrated in figure 16 was designed to determine the effect of high intensity light on the phototropic system by studying the system's behavior following the high intensity exposure. In these cases the plants were exposed twice to light. The first exposure was one of those described just above, a dosage of 31,600 MCS, and conditions known to produce little or no response. The second exposure was 1,000 MCS, at which dosage failure of the reciprocity law does not occur, and a consistent tip curvature response is always obtained. The two horizontal lines indicate the curvatures of controls exposed separately to one or the other of the dosages described above. The curve between illustrates the results of double exposure, with the abscissa representing the time between exposures. This time was always measured from the end of the initial exposure to the beginning of the final one. For 2 minutes following the first exposure the second is completely ineffective and a total refractory period is observed. For approximately the next 18 minutes the plants gradually recover their phototropic sensitivity (tip curvature in this case) and pass through a partial refractory period. Twenty minutes after the initial exposure the final one is again completely effective, and the photosensitive system for tip curvature has apparently returned to normal. Almost identical results were obtained with Avena.

Thus a light dosage of 31,600 MCS administered to corn coleoptiles in 2 minutes does cause changes in the tip curvature photosensitive system unlike those obtained with the same dosage administered at one-tenth the intensity and ten times the duration. Not only are these changes ineffective in bringing about a curvature response, but, following them, the system has completely lost its normal phototropic sensitivity. Twenty minutes are required to recover normal sensitivity. A limiting dark reaction therefore can not be considered as the sole reason for failure of the reciprocity law, and pigment inactivation by high light intensity provides an alternative possibility.

Discussion

The Bunsen-Roscoe reciprocity law is valid for phototropic curvatures, but only within a closely circumscribed range of dosages. A survey of the literature reveals that at threshold light dosages, a curvature response is dependent only upon the total energy impinging on the system, and is independent of the manner in which the light is administered (4, 19, 20). Furthermore, both Arisz (1) and Haig (23) clearly showed the law to be valid between first perceptible curvature and maximum tip curvature for the oat coleoptile. The present results indicate that with the lowest light dosage that will induce maximum first increment curvature of corn and first positive curvature of oat coleoptiles, the response depends only upon total energy unaffected by reciprocal variations of intensity and time. Unfortunately, Haig extended his observations only to maximum tip curvature, apparently the limit of complete validity of the reciprocity law according to the results above, but not beyond.

The investigations of Arisz (1) on the other hand, show that such validity is lacking in the case of negative curvatures of Avena coleoptiles. Arisz was able

to obtain clear negative curvatures at the appropriate dosages only with a light intensity of more than 12 meter candles. Below this intensity, curvatures were either indifferent or distinctly positive. Although a discrepancy exists between his results and those of the present study as to the exact intensity at which negative curvature is first observed (see fig 13), both studies are apparently dealing with the same phenomenon and the conclusion that the reciprocity law is of limited applicability is the same.

The earlier authors were clearly aware of the lack of validity of the reciprocity law at high dosages of light. Thus the concept of an effect of light on the Stimmung or sensitivity (tonus) of the phototropic system(s) arose. When plants are given a dosage of light from all sides, their subsequent sensitivity to a unilateral dosage is clearly reduced, the amount of reduction depending upon the magnitude of the preillumination. Arisz (1) investigated this matter in detail and subsequent authors gave considerable thought to its explanation (10, 16, 28). Thus a high intensity unilateral illumination might itself reduce the phototropic Stimmung in such a way that the responsiveness of the phototropic system to the unilateral illumination might be reduced, and the reciprocity law would be invalid. On the other hand, should the same dosage of unilateral light be administered at a low intensity, it seems possible that a reduction in Stimmung might well not occur until phototropic induction was complete, if it indeed occurred at all, and the reciprocity law would appear valid.

The Weber-Fechner law states that when an organism is subjected to two stimuli (presumably opposing) of different intensity, the response is not proportional to the absolute difference between the two, but rather to their ratio. Pringsheim (25) demonstrated the validity of this law for the reaction threshold of phototropic curvature using opposed light sources and Avena coleoptiles, and Fuller and Thuente (20) confirmed it for a number of different plants and a number of different dosages. Thus if the opposing dosages were large, the absolute difference between the two had to be far larger to give threshold curvature than if they were both small, but in both cases the ratio was the same. In this case also, one is forced to the conclusion that sensitivity to unilateral light is reduced by light itself, and that the reciprocity law could hardly be considered valid.

There are several papers concerned with the effect of intermittent light stimuli as compared with continuous ones of the same total energy. Nathansohn and Pringsheim (24) investigated a number of different plants and found that intermittent stimuli produced the same response as the comparable amount of energy given continuously, and thus the law of Talbot, or the summation of stimuli was valid. Miss Barbara Hill (pers communic) has demonstrated in our laboratory that for a number of different dosages, as long as light intensity is sufficiently low that maximum curvature response is obtained, intermittent light induces the same curvature as continuous light of comparable energy, in corn coleoptiles. Both of the above cases, however, involved light and dark periods of fractions of a second. In two cases, with longer dark and light periods (several seconds or actually minutes) intermittent light did not induce curvature comparable to that obtained from continuous light. Günther-Massias (22) found that with both *Avena sativa* and *Sinapis alba* very low dosages of light (69 and 139 MCS, resp) were more effective in inducing curvature if administered in brief flashes of several seconds, separated by comparable or slightly longer dark periods. The optimum ratio of light to dark periods was either 1 : 1 or 1 : 2, in both cases, with an intensity of 0.23 meter-candles. Here failure of the reciprocity law does not relate directly to reduction in phototropic sensitivity or responsiveness as a result of light, and thus is not comparable to the cases discussed above. This rather special phenomenon requires further investigation.

However, a case described by Du Buy (14) is of particular interest, since what is considered as apparent failure of the reciprocity law appears open to the converse interpretation. Du Buy irradiated oat coleoptiles with monochromatic light for two periods, separated by varying amounts of time. The sum of the two energies was always at some position along the first positive curvature peak (see fig 1, log MCS 1–4), sometimes to the left and sometimes to the right of it. If the dark interval was short the two irradiations induced the same curvature as if the illumination had been continuous, as might be expected. If, however, the interval was long (several minutes) the curvature response was usually different. When the sum of the two energies would be expected to give a response beyond the maximum peak (hence in the range where present data indicate failure of the reciprocity law to occur) a separation of 15 minutes resulted in maximal curvature, rather than submaximal. Under those conditions curvature which would be expected if the reciprocity law were valid was obtained. Du Buy felt that since he did not get the submaximal curvature expected from the continuous dosage, following wide separation of stimuli, the maximal curvature obtained represented a failure of the reciprocity law. The present interpretation is precisely the opposite: the reciprocity law is valid with wide separation since maximal curvature, expected only normally with a lower intensity and longer exposure, was obtained. With short separation, the lower than maximal curvature obtained should represent failure of the reciprocity law.

The above discussion illustrates the danger of assuming complete validity of the reciprocity law under all conditions, and figure 8 illustrates this danger. In the experiment shown, it was possible to obtain three distinct results from intact and non-preilluminated coleoptiles from continuous illumination without varying the dosage: first, with low intensity, substantial curvature, presumably resulting from two separate mechanisms (lateral transport and some type

of light-growth reaction); second, with higher intensity, failure of both of these mechanisms and no response at all; and third, with extremely high intensity, a curvature response apparently mediated by a third and entirely distinct mechanism. Actually even in the low intensity range within which the reciprocity law is valid, there is at present no evidence to suggest that the relative contributions of the first two mechanisms mentioned above are always the same.

It has already been pointed out that the tip of the Avena coleoptile is about 1,000 times as sensitive to unilateral light as the base (26). Data presented above indicate the same situation to be found in the corn coleoptile. A dosage between 50 and 100 MCS is sufficient to elicit first perceptible tip curvature, whereas a dosage above 10,000 MCS is required to obtain any base response, and hence the beginnings of second increment curvature (see fig 10). Brauner (8) has done experiments similar to those of Boysen-Jensen (6), Boysen-Jensen and Nielsen (7), and those reported above, utilizing a small fragment of a cover slip inserted into a slit in the Avena coleoptile tip either parallel or perpendicular to the plane of the light beam. Brauner's results demonstrate that as the light dosage is gradually increased, the relative importance of the tip reaction declines while that of the base reaction increases. He feels that the tip reaction is the first to appear, developing rapidly, while the base reaction develops more slowly. Since his dosages are rather high (above 300,000 MCS) and the range of validity of the reciprocity law is not known for Avena at these dosages, it is difficult to compare Brauner's results with those of the present study. Nevertheless, his results provide further evidence for two separate phototropic reactions. Experiments to be described elsewhere suggest that for corn coleoptiles these two reactions involve entirely separate mechanisms, and Went's review (32) discusses in detail the various physical differences between the two responses. In the face of this accumulated evidence, there seems no reason to expect that both mechanisms are necessarily mediated by the same pigment system. Two separate pigment systems, as discussed by Went (32) are equally plausible.

The results of the flashing light experiments described above, combined with those utilizing two separate dosages with a varying interval of time between them, suggest a reasonable hypothesis to account for the failure of the reciprocity law, a phenomenon perhaps at least partially equivalent to the decrease in Stimmung described by the earlier authors. Photoinactivation of receptor pigment molecules possibly occurs under conditions of high light intensity. However, such inactivation must happen in two stages, the first of which is normal activation, inducing curvature, and the second of which is another photochemical event involving the already activated molecules, resulting in response failure. Since the experiments described above were done using white light, it might well be argued that with increasing light intensity, there is an increasing amount of photoinactivation of pigment molecules by wavelengths other than those normally inducing curvature. Such a suggestion would eliminate the necessity of assuming more than one stage of pigment reaction to light. However, preliminary experiments using monochromatic blue light, obtained from a mercury arc by means of Corning filters, indicate that the reciprocity law fails with increasing intensity of monochromatic light just as it does with white. Indeed, this conclusion is implicit in the results of Du Buy's experiments (14), described above, which also utilized monochromatic light. There remains therefore, the possibility of photoinactivation of receptor pigments by the same wavelengths effective in inducing curvature. Thus when high intensity light is administered in flashes of less than a certain length, only the first stage, suggested above, would occur, but not the second, and a normal response would be observed. The dark period might allow activated pigment molecules to dissipate their briefly stored energy to a phototropic response system and return to their pre-activated state. If, under the conditions of these experiments, light flashes were longer than a certain limiting minimum, the second stage might occur with some pigment molecules, resulting in their inactivation, and therefore in a less-than-maximum response. If the light were continuous, or the flashes excessively long, inactivation of all pigment molecules might occur, and the 20-minute refractory period would then represent the time required either for them to be returned to their original pre-activated state or for de novo synthesis of receptor pigment, should the inactivated molecules be permanently destroyed.

There is, of course, an alternative hypothesis that at least for tip curvature, high intensity light does not affect the pigment system at all, but rather in some way affects the lateral transport system. In this case, negative curvatures such as those found in Avena coleoptiles would be mediated by some mechanism other than lateral transport, and the hypothesis suggested above to account for negative curvature would be invalid. However, the results of Asana (2) and Wilden (34) indicate that following exposure of Avena coleoptiles to dosages of light which elicit negative curvature, there is indeed an auxin differential between light and dark sides, but that the highest amount of diffusible auxin is obtained from the lighted side—the reverse of that described for positive curvature (12, 31). Asana further suggests that the amount of auxin obtained from the illuminated coleoptiles is not significantly lower than that obtained from plants kept in darkness, and thus auxin inactivation or inhibition of synthesis as the cause of the auxin differential are effectively ruled out. Thus the lateral transport system may well be functioning but in the reverse direction from that observed during positive curvature. The first hypothesis presented above, concerning pigment inactivation, seems more reasonable than the alternative in providing an explanation for failure of the reciprocity law.

There must certainly be a dark reaction following normal pigment activation, since phototropic induction and curvature are frequently separated in time. Indeed, results from intermittent light experiments, obtained by varying the length of the dark period under different conditions of dosage and temperature may well provide a measure of such a reaction, and experiments of this nature are in progress. It seems clear that the reciprocity law becomes invalid with increasing light intensity as a result of pigment inactivation, however, rather than as a result of limits imposed by a thermochemical dark reaction.

Two recent papers (3, 5) have suggested that preillumination of coleoptiles with red or orange light may significantly influence their subsequent phototropic sensitivity. Preliminary experiments indicate that with corn coleoptiles, virtually no tip curvature at all can be obtained without previous irradiation with red light. Since all of the experiments described above were performed under dim red light, it was important to determine the relationship between the reciprocity failure described above and red light irradiation. Miss Barbara Hill (pers communic) has shown that as long as a sufficient red light exposure has been made, the reciprocity failure curves obtained are virtually the same as those described above. Fortuitously, then, the experiments described in the present paper were done under optimum conditions.

The results presented above suggest that in corn coleoptiles there are at least three types of phototropic responses. At least one of these must involve lateral translocation of auxin or a precursor (12). The nature of the other two responses, in terms of the role of auxin, will be considered in another paper describing detailed growth hormone studies.

Summary

The Bunsen-Roscoe reciprocity law is valid for the phototropic curvature of corn and oat coleoptiles in response to unilateral white light only for light dosages of 1,000 meter-candle-seconds or less. The pattern of failure of the law at higher dosages is described in detail for corn and in brief for oats. A dosage-response curve for corn coleoptiles, obtained by administering all dosages under conditions of intensity and time within which the law is valid, differs considerably from that described in the literature for oat coleoptiles. It is step-shaped, with a first and second increment corresponding to the first and second positive curvature peaks of the older curve. First increment curvature is tip curvature, while second increment curvature is a combination of both tip and base curvature. It is suggested that the earlier curve is an artifact resulting from excessively short exposure times at high light dosages.

Negative curvature of oat coleoptiles is interpreted in terms of failure of the reciprocity law. It is thought that under conditions producing negative curvature, the phototropic system on the illuminated side of the coleoptile fails completely, but that enough light penetrates the coleoptile to activate the system on the shaded side, thus reversing the normal situation and producing curvature away from the light source.

Evidence is presented suggesting that reciprocity failure in corn coleoptiles, at least at one dosage (31,600 meter-candle-sec), is the result of pigment inactivation, rather than of some limit imposed by a thermochemical dark reaction. The most reasonable hypothesis to account for this inactivation is that under conditions of high light intensity, a pigment molecule may become activated by light and then excited once again before sufficient time has elapsed for it to dissipate its briefly-stored energy to the phototropic response system. The second excitation results in inactivation of the pigment and a failure of curvature induction. If the phototropic system is completely inactivated in this manner, 20 minutes are required either for a return of the pigment molecules to their original pre-activated state or for de novo synthesis of pigment molecules to replace those inactivated, under the experimental conditions used.

Acknowledgments

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RELATIONSHIPS BETWEEN TIME COURSE, CHROMATIC TRANSIENT, AND ENHANCEMENT PHENOMENA OF PHOTOSYNTHESIS[1]

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A previous report (11) presented a confirmation and extension of the work of Blinks (1, 2) on chromatic transients which accompany wavelength changes in the incident light and of the work of Emerson and co-workers (4, 5) on the increase in effectiveness of long wavelength red light (700 mμ) by added light of shorter wavelength. The almost identical action spectra of the two phenomena were taken as a demonstration of a specific participation of chlorophyll b in both effects. A third effect was noted but not elaborated: the dependence of time course of photosynthesis upon wavelength of incident light. Further study based upon the time courses reveals that there are reactions specifically associated with chlorophylls a and b which have markedly different time constants.

METHODS

The methods are not essentially different from those of the previous report (11) and will be presented in summary only. Rate of photosynthesis was measured by the platinum electrode method of Haxo and Blinks (7) as modified by Dr. Haxo for use with unicellular algae. The platinum strip was covered by a cell layer about 15 μ thick of *Chlorella pyrenoidosa* Chick and separated from a large reservoir of nutrient solution by a dialyzing membrane. The diffusion characteristics of the system are such that the oxygen diffusion current is a direct measure of net rate of oxygen evolution. The amplified current was recorded on a strip chart recorder with a pen speed of 1 second full scale. The recorded current difference produced by a single light beam was linear with light intensity over the range of intensities available. Illumination was provided by two light beams, one from a grating monochromator of half band-width 5 mμ and the other via an interference filter (referred to as 700 mμ) of peak transmission 702 mμ and half band width 11 mμ. The temperature, unless otherwise specified, was within the range 22 to 25° C.

Both light beams were controlled by electrical shutters which permitted manual or automatic timer operation. For one of the experiments close synchronization of the shutters was required for alternating the two light beams at short periods. The shutters were adjusted and their characteristics observed on an oscilloscope which sensed directly the signal from a silicon photocell placed just above the electrode. Both shutters required about 0.03 seconds from beginning to completion of opening and closing. Since the shutters were actuated from the same relay their synchronization was accomplished by positioning with respect to the light beams. Adjustment was not perfect but was such that at each half-cycle there was a small increase and on the other half-cycle a small decrease in light as compared to the steady level. These were estimated individually as amounting to less than 3 %, with the net error considerably less, at the shortest period of 0.6 seconds and correspondingly smaller at the longer periods.

TIME COURSE OF PHOTOSYNTHESIS VS WAVELENGTH. Upon illuminating the electrode with its covering algal film the rate of net oxygen evolution, as measured by the oxygen diffusion current, increases toward a steady value. With the electrode and chamber used, the time required to obtain a steady rate of photosynthesis was 2 to 6 minutes although 50 % of the maximum rate usually was reached in 15 seconds or less (cf. fig 1). Initially we were hesitant to study time course phenomena since we have found no way to measure the time lags of the electrode, if any. Further, we were disturbed that the complete response times observed appear to be much slower than those reported by Brackett et al (3) using a pulse polarized electrode and those of

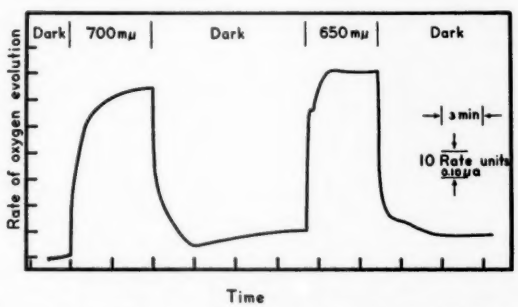

FIG. 1. Time course for rate of oxygen evolution at 650 and 700 mμ. Tracings of records made at slow chart speed.

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Whittingham (13) using a photometric method with hemoglobin. However, three characteristics have provided some degree of confidence. First, the initial response in the first few seconds of light is very rapid. Second, the initial decrease in rate on darkening is even more rapid than the increase upon illumination. And, third, the time course of change in rate is wavelength-dependent. We proceeded to examine the wavelength effects in the belief that the differences in time course observed cannot be greater than those actually occurring in the cells, whatever lags the measuring system may contain.

The most dramatic effect of wavelength upon time course is manifest within the first 15 seconds. Experiments such as that presented in figure 2 were obtained by recording at a rapid chart speed of 4 inches/minute and translating the data to per cent of maximum rate vs time. The curves of different experiments showed considerable variation, attributed to unknown factors in the previous history of the cells, but were not much affected by the light intensity used nor by the length of the preceding dark period, at least within the range of 4 to 10 minutes. Two important characteristics shown in figures 1 and 2 were observed without exception: A: The initial decay in the dark is more rapid than the initial rise in light, and B: *both* the initial rise and initial decay are more rapid at 650 than at 700 mμ.

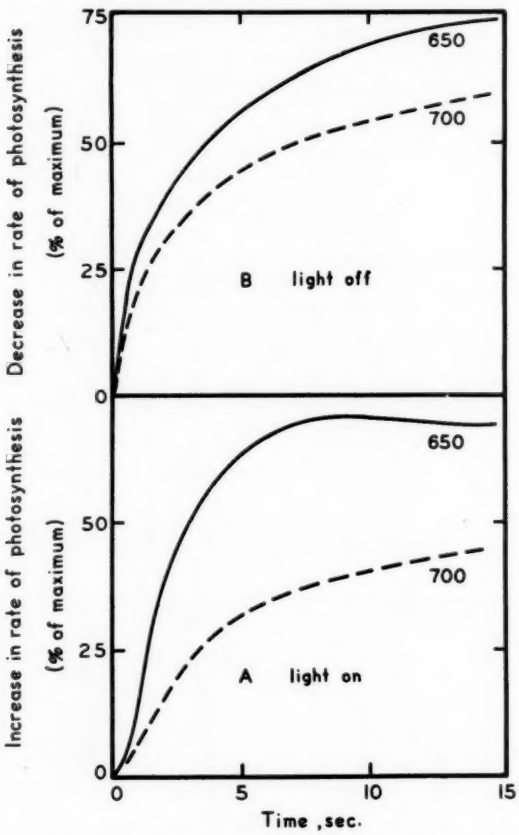

FIG. 2. Time course curves for rate of oxygen evolution over the first 15 seconds for 650 mμ and 700 mμ. A: Rise in rate following the dark → light transition. B: Decay in rate following the light → dark transition. Taken from recordings at 4 inches/minute chart speed and presented as per cent of maximum rates which were 61 rate units for 700 mμ and 59 rate units for 650 mμ.

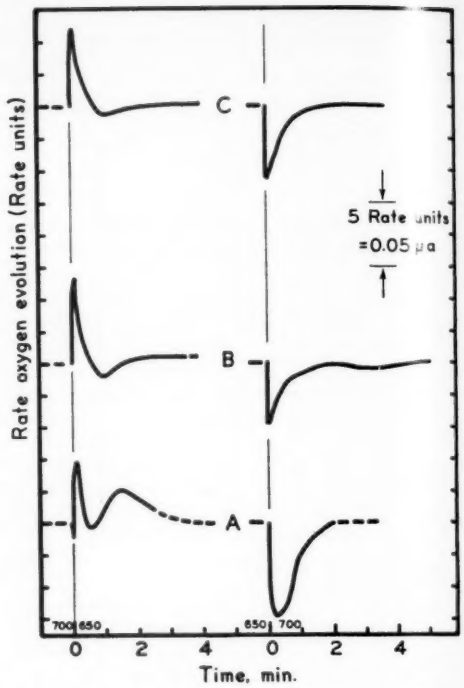

FIG. 3. The 700 → 650 mμ and 650 → 700 mμ chromatic transients. A: As constructed from the rise and decay curves of figure 2 (see text). B: As constructed from the rise and decay curves of figure 4 (see text). C: As observed (traced from the same record used for fig 4).

RELATION OF CHROMATIC TRANSIENTS TO TIME COURSE. Very early in our work it appeared that the transients were related to the time course effects. A spike or marked inflection on the time course curve (fig 1), often (but not always) observed at about 12 seconds after the beginning of 650 mμ illumination, had some of the characteristics of the 700 → 650 mμ transient (fig 3, C). A clear relationship between the two could be developed. If, after observing the transients with matched beams at 700 and 650 mμ, the intensity of the 700 mμ beam was decreased stepwise in successive periods, the 700 → 650 mμ transient developed into the spike of the 650 mμ time course curve.

A simple hypothesis for the chromatic transients is that they represent the net effect of simultaneous rise and decay curves. The 700 → 650 mμ transient might result from the rise in rate of a reaction associated with 650 mμ added to the decay in rate of a reaction associated with 700 mμ. The 650 → 700 mμ transient might result from the opposite combination. The hypothesis may be tested by attempts to synthesize the transients from rise and decay curves observed under appropriately chosen conditions. A first attempt is shown in figure 3A. The 700 → 650 mμ transient is taken as the numerical difference at any time between the 650 mμ rise curve and the 700 mμ decay curve of figure 2; likewise, the 650 → 700 mμ transient is taken from the 700 mμ rise and 650 mμ decay curve. Even the rough similarity of figure 3A to the experimentally observed curve of figure 3C is remarkable since the transients of figure 3C are observed under conditions in which light is not turned on or off but merely changed in wavelength.

In search for a closer simulation of events occurring during the 700 → 650 mμ and the 650 → 700 mμ transitions we make the following simplifying assumptions: A: At 700 mμ all incoming light quanta are absorbed by chlorophyll a; B: At 650 mμ a fraction, R, of the quanta is absorbed by chlorophyll a and a fraction, 1 — R, by chlorophyll b; C: The operation performed by chlorophyll a is the same whether quanta are absorbed at 650 or 700 mμ. If light beams at the two wavelengths are adjusted in intensity to give equal rates of photosynthesis then the 700 → 650 mμ transition should be accompanied by a sudden input of (1 — R) quanta to chlorophyll b, a sudden decrease of (1 — R) quanta to chlorophyll a, and a fraction R of the quanta to chlorophyll a unchanged by the transition. The 650 → 700 mμ transition should be attended by a reversal of these changes.

Figure 4 presents an experiment designed to observe rise and decay curves under the conditions noted above. The fraction R, which must be estimated empirically, was set at 0.68 for this particular experiment by the transmission of a screen inserted at point C and removed at F. In figure 3B transients are synthesized from the rise and decay curves of figure 4. The 700 → 650 mμ transient is constructed by adding together the decay curve following C and the rise curve following D; likewise, the 650 → 700 mμ transient is constructed from the decay curve following E plus the rise curve following F. There is good agreement both in contour and in peak heights between the synthesized transients of figure 3B and the transients actually observed within the same experiment (fig 3C and fig 4, A, B.).

In other experiments similar to that of figure 4 we varied the screen transmission to simulate values of R of 0.48 to 0.74. The best fit of the subsequently synthesized transients is that shown for R = 0.68. The procedure is sensitive to small drifts occurring during the experiment and is not a precise method for establishing a value for R.

We recognize that complete explanation of the transients is probably more complex than suggested by our simplifying assumptions used in searching for appropriate conditions for observation of time course curves. Moreover, our argument has implied that R should represent the fraction of incoming quanta absorbed by chlorophyll a. We did not measure light absorption nor quantum efficiency. Hence our empirically chosen value of R was based upon the sustained rate of oxygen evolution determined by incident intensity, absorption, and quantum efficiency. Since the several contributions to the empirical value of R cannot now be resolved, no significance can be assigned even if it were measurable with precision. And the assumptions made in setting up the experiment of figure 4 are not necessarily confirmed by the results. However, these considerations are beyond the original objective. The ability to synthesize transients in reasonable facsimile from rise and decay of appropriately chosen time course curves is convincing evidence for the relation between time course and transient phenomena.

One other characteristic of the chromatic transients should be noted since it has bearing on the action spectra previously presented (11). The transient peak height, calculated as a per cent of steady-state photosynthesis, is not quite independent of the steady rate (cf. table I). We cannot determine whether this characteristic reflects a limitation in time response of the electrode and recorder or whether it describes an inherent character of the transients themselves. In the latter case some, although possibly very small, effect of intensity on time course curves such as those of figures 1 and 2 is implied. In any event there is possible concern about the differences between maxima and minima of the action spectrum, figure 4, A (11), which may be somewhat greater than those shown.

TABLE I

MAGNITUDE OF CHROMATIC TRANSIENTS VS LIGHT INTENSITY

RELATIVE INTENSITY	STEADY RATE* PHOTOSYNTHESIS	700 → 650 mμ UP TRANSIENT		650 → 700 mμ DOWN TRANSIENT	
		PEAK HGT*	% STEADY RATE	PEAK HGT	% STEADY RATE
100	87	22.5	26	10.5	12
68	60	17.5	29	9.5	16
34	29	11	38	7	23

* In relative rate units. One rate unit is a difference current of 0.01 μamps.

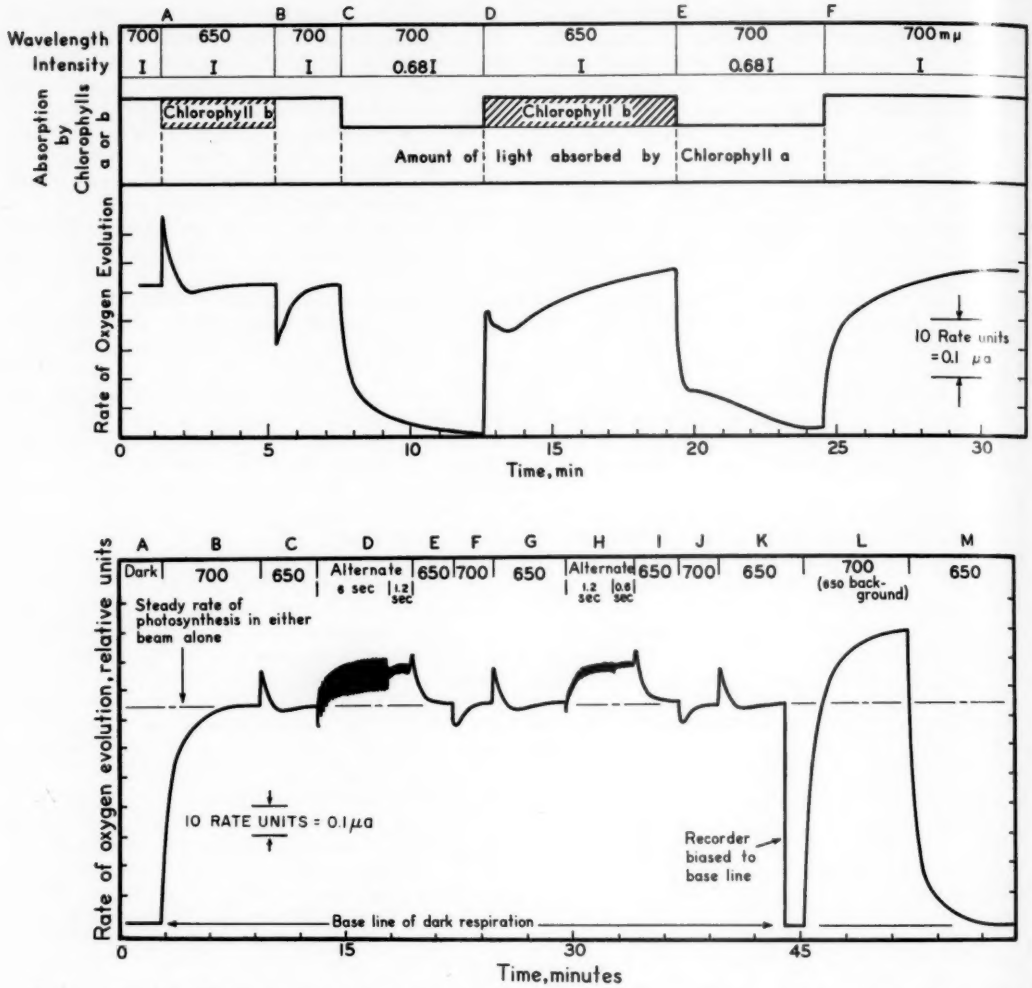

FIG. 4. (*Upper*) Chromatic transients and the rise and decay curves selected for their synthesis. A tracing of the original record obtained in an experiment with two light beams at 650 and 700 mμ matched in intensity, I, to support equal rates of photosynthesis. The two beams were alternated or screened at the transition points noted as follows: A: 700 → 650 mμ; B: 650 → 700 mμ; C: Insert wire screen of transmission 68 % in the 700 mμ beam; D: 700 mμ, intensity 0.68 → 650 mμ; E: 650 mμ → 700 mμ, intensity 0.68; F: remove 68 % screen from 700 mμ. The lower block diagram indicates the presumed rate of quanta input to chlorophylls a and b (see text).

FIG. 5. (*Lower*) Enhancement of photosynthesis between two light beams of 650 and 700 mμ. A reconstructed tracing from a single experiment. The illumination during each time period is indicated at the top of the record. In periods D and H the two light beams were alternated to give 0.6, 1.2, or 6.0 seconds of each beam as noted.

RELATION OF CHROMATIC TRANSIENTS TO ENHANCEMENT. The time course curves and chromatic transients imply that the reactions specifically associated with wavelengths 650 and 700 mμ have time constants differing in values by seconds. Therefore it should be possible to obtain enhancement between 650 and 700 mμ light beams even when the two beams are separated in time by rapid alternation. The expectation was confirmed.

Figure 5 presents a reconstruction from tracings of the original record of an experiment made with light beams of 650 and 700 mμ matched in intensity

to support equal rates of steady photosynthesis at a level of 74 rate units.

Alternation of the two beams at periods of 3 to 6 minutes gives rise to the typical transients each followed by a complete return to the steady rate. The alternated periods of the 650 and 700 mμ beams could be reduced in length by a series of timers each with a precisely adjusted 50 % cam. In section D of the record the 650 and 700 mμ beams are alternated to give 6 second periods of each. The individual character of the transients is lost and the transients appear as nearly symmetrical curves. The important point is that the entire envelope is above the steady rate of photosynthesis supported by either beam alone. Since the curves within the envelope are nearly symmetrical, the center of the envelope represents the average rate of oxygen evolution supported by the alternating beams. The height of the center of the envelope above the steady rate line measures the degree of enhancement. As demonstrated in this way the enhancement becomes clearly apparent at 15 second periods (not shown in this part of the record and approaches a maximum at our shortest period of 0.6 seconds. For comparison the enhancement as observed for 700 mμ superimposed upon continuous background of 650 mμ may be seen by comparing sections B and L of figure 5.

The point clearly established by figure 5 is that two matched light beams at 650 and 700 mμ alternated at short periods give a greater rate of net oxygen evolution than can be supported by either beam alone. From inspection of figure 4 it is apparent that the amount of enhancement in alternated beams (period H) is only about half that obtained when the beams were superimposed (period L). However, it must be noted that only half as much light was presented by the alternated as compared to the superimposed beams. The quantitative characteristics taken from the experiment partly shown in figure 5 are summarized in table II. It has been shown (11) that the relative magnitude of enhancement is independent of total light intensity but governed only by the 650/700 mμ intensity ratio. Hence the relative enhancement observed in alternating light beams may be compared with that observed conventionally with the two beams superimposed.

As previously discussed (11) enhancement may be reckoned in either of two ways. When referred to the total light presented (650 plus 700 mμ) the enhancement factor for 0.6 second alternation (table II and period H of fig 5) is calculated as $87.5/74 = 1.18$. By comparable calculation the enhancement factor for the two beams superimposed (table II and period L of fig 5) is identical, $(101 + 74)/148 = 1.18$.

Enhancement may also be calculated on the assumption that the entire gain is due to increase in efficiency at 700 mμ alone. When the two beams are presented simultaneously enhancement, referred to the 700 mμ beam alone, becomes $101/74 = 1.36$. By similar calculation for the 0.6 second alternation, and noting that the 700 mμ beam is presented only half the time, enhancement by similar calculation becomes $(87.5 + 13.5)/74 = 1.36$.

The quantitative comparison is even more remarkable than the qualitative demonstration. *The interaction between 650 and 700 mμ, as measured by relative increase in rate of oxygen evolution, is the same whether they are presented continuously or alternated at equal periods of 0.6 seconds.*

We also examined by the technique demonstrated in figure 5 the effects of rapid alternation of two beams at 630 mμ, presented by the monochromator, and 647 mμ presented via an interference filter of 11 mμ half band width. As noted previously (11), these two beams give the chromatic transients but do not show measurable enhancement. Rapid alternation of the two beams at 1.2 and 0.6 seconds (as in period H, fig 4) gave rise to envelope centers displaced less than one rate unit above the steady rate line and an enhancement factor, calculated as in table II, of not more than 1.02.

Effects of Temperature. In order to study effects of temperature, the solution reservoir was reduced in size to 70 ml and provided by a jacket at the sides and bottom through which constant temperature water could be pumped. The decrease in volume and in the gas-liquid interface area made it necessary to bubble aerating gas continuously in order to avoid carbon dioxide or oxygen depletion. An extreme temperature of 2° C was chosen and 0.5 % instead of 5 % carbon dioxide was used to avoid possible toxicity of carbon dioxide due to increased solubility at the low temperature. No differences in the transients or steady rate of photosynthesis between 0.5 and 5 % carbon dioxide were detectable at 22° C.

The essential question of the temperature study was whether or not the transients are temperature sensitive. On this point the following statements may be made. In two experiments the height of the 700 → 650 mμ transient, estimated as per cent of the steady rate, decreased from 18 to 14 % and from 16 to 9 % for the temperature change 22 to 2° C.

Table II

Enhancement in Alternating 650 and 700 mμ Light*

Time period of each beam Sec	Avg rate oxygen evolution rel. rate units	Enhancement factor**
(long)	74	...
15	79	1.07
6	84.5	1.14
1.2	86.5	1.17
0.6	87.5	1.18
Simultaneous	175***	1.18

* (Taken from the experiment partly shown in fig 4)
** Rate of oxygen evolution in alternating light divided by rate (74) supported by each light beam alone when the periods are long.
*** Discussed in text.

The transients were extended over longer time periods so that on an encompassed-area basis the differences would have been even smaller. In short, the transients are temperature-sensitive but with a relatively small temperature coefficient.

Intensive temperature studies were not pursued, since we were unable to obtain a value for the temperature coefficient of electrode sensitivity. However, two observations are of some interest. The time course curves presented very slow rise and decay in rates of oxygen evolution such that periods of 15 minutes or longer were required to complete both the dark-light and light-dark transitions. In spite of this the characteristically more rapid rise curve at 650 mμ persisted although the decay curves at 650 and 700 mμ were indistinguishable.

A second observation arose from our procedure of making the temperature change 22 to 2° C while the cells were exposed to alternating light of 650 and 700 mμ at 2 minute periods as controlled by an automatic timer. Invariably the rates of oxygen evolution, which had been balanced in the two beams at 22° C in order to measure the transients, became unbalanced as the temperature dropped; at 2° C the rate of oxygen evolution at 700 mμ always became less than at 650 mμ. The differential temperature coefficient 700/650 for the temperature change 2 to 22° C was a factor of about 1.3. We checked the light intensity curves at 2° C. We could approach light saturation at 650 mμ but not at 700 mμ with the light sources used. The differential effect of temperature at 650 and 700 mμ is not due to any differential light saturation. The temperature effect observed is opposite to that reported by Emerson et al (5). They reported that quantum yields at long wavelength increased at low temperature (5° C). It is conceivable that our observations, made only with respect to incident rather than absorbed light, are due to a slight sharpening of the chlorophyll a absorption band and decreased absorption of 700 mμ at lower temperature.

Discussion

The phenomena of wavelength dependency of time course, chromatic transients, and enhancement in terms of a common but incomplete model must be based upon a reaction in some way specifically associated with chlorophyll b. The time course and transient effects may arise because this reaction of chlorophyll b allows more rapid adjustment in the steps required for oxygen evolution. Enhancement could arise because this reaction allows more efficient utilization of energy from chlorophyll a. A flaw in this generalized model is that experimentally enhancement appears to be tied to both chlorophyll b absorption and to the wavelength region 685 to 710 mμ while the transients are tied only to chlorophyll b vs chlorophyll a absorption. Possible qualifications of this anomaly have been noted (11).

The explanation of the chromatic transients in terms of rise and decay of time course curves is self-consistent since both phenomena contain equal contributions of the unknown time lags of instrumentation. However, the unknown time resolution of our records gives rise to considerable uncertainty in estimating real reaction times from any observations of changes in rate of oxygen evolution. It is fortunate that the demonstration of enhancement in alternated light beams becomes independent of any properties of time resolution of instruments. In sections D and H of figure 5 the vertical width of the envelope but not the vertical position is affected by time lags of measurement. The phenomenon should be observed equally well by manometry.

The time constants of the postulated reaction following chlorophyll b absorption can be specified only approximately from the data available. The intermediate(s) involved must reach rate-saturating concentrations in fractions of a second and have lifetimes measured in seconds. It is tempting to speculate that the reaction also is involved in the long-flash phenomena observed in high-intensity intermittent light (8). The time constants required appear close to those demonstrated by Whittingham and Brown (14). They observed an increase in yield from a single long flash when it was preceded by a short flash. When the flash separation was varied the extra yield of oxygen reached a maximum at a spacing of 0.7 seconds and remained appreciable for spacings as long as 10 seconds. It now appears that it would be useful to examine the effect of wavelength of background light upon flash phenomena.

Assignment of the postulated reaction to chlorophyll b, or perhaps to accessory pigments in general, leaves unspecified the locus of the reaction. If we assign the location to the pigment complex itself, then we are faced with long time constants inconsistent with the lifetimes of excited states. However, the rise and decay times for the photo-induced electron spin resonance in photosynthetic material are in agreement in order of magnitude (12). If, on the other hand, we assign the reaction to the aqueous phase enzymatic sequence, then a separate reaction chain arising from chlorophyll b (or other accessory pigment) is required. This is hardly consistent with the evidence of highly efficient energy transfer from accessory pigments to chlorophyll a.

In quite separate context from the above problem, but implicitly requiring a separate enzymatic sequence, Blinks (2) has proposed that the transients arise from changes in level of respiration. He suggested that the transient as 700 → 650 mμ arises from an increased respiration which starts a little after photosynthetic increase, thus giving the initial peak; a persistence after return to longer wavelength would give the depression of the 650 → 700 transient. We have found no evidence for Blinks' hypothesis nor any unequivocal evidence against it. The burden of evidence is to the contrary. We have observed occasional, but by no means consistent, temporary increases in respiration level following illumination at 650 mμ. In the 490-460 mμ region we are always troubled by drifts toward increased respiration

similar to those noted by Emerson and Lewis (6). However, neither of these effects approaches the transients either in consistency or in magnitude. There are several considerations which speak against the hypothesis: A: The increase in respiration at 650 mμ would have to be large, about ¼ of the apparent photosynthetic increase as judged by the height of the 700 → 650 transient; B: The actual quantum yield at 650 mμ would have to be ¼ higher than at adjacent wavelengths such as 630 mμ; C: Our rise curves following illumination are much faster at 650 than at 700 mμ and remain so even if calculated in percent of a final rate ¼ greater; and D: The temperature coefficients for the transients is small. For these reasons we prefer to search for a reaction of some character other than that of a respiratory process closely geared to the photosynthetic sequence.

It is possible to place some broad specifications upon the nature of the reaction necessary to account for the effects observed. It is associated with absorption of quanta by chlorophyll b (or other accessory pigment). It must accomplish a faster adjustment of the photosynthetic mechanism manifest both on increase and on decrease in illumination. It must permit more efficient transfer or trapping of quanta absorbed by chlorophyll a at long wavelength. It must have intermediates with lifetimes measured in seconds.

A possible basis of explanation lies in the observations of Kok (10) on a reversible absorption change at 703 mμ. The absorption of a suspension of blue-green algae at 703 mμ was increased by red (>600 mμ) but decreased by far red (>670 mμ) light presented in repeated high intensity flashes. The 703 mμ pigment previously (9) had been postulated as a likely candidate for the final photoreceptor in photosynthesis. Extension of the observation leads to a model in which the 703 mμ pigment is changed to a converted form by light quanta absorbed directly or via chlorophyll a. Reconversion of the 703 mμ absorbing form might take place slowly in darkness or rapidly following transfer of quanta directly from accessory pigment. Kok's model is based upon limited data, and further extrapolation to explain our results is not warranted. However, the possibilities raised make us hesitant to attempt explanation of the apparent specific role of accessory pigment in the conventional terms of known reactions.

Summary

Possible relationships were sought between the wavelength dependent phenomena of time course, chromatic transients, and the Emerson effect of enhancement. Rate of oxygen evolution was measured as the current from a polarized platinum electrode covered by a thin layer of Chlorella. Illumination was provided by one or both of two monochromatic light beams at 650 and 700 mμ.

It was possible to demonstrate a close relationship between the chromatic transients and time course phenomena. Good approximations to the transients could be constructed from the rise and decay curves in rate of oxygen evolution observed under appropriate conditions. By decreasing the period of alternation of the two light beams an orderly progression could be demonstrated between the chromatic transients of Blinks and the Emerson effect of enhancement. When the two light beams were alternated at short periods (0.6 seconds) the relative magnitude of enhancement was equal to that observed when the two beams were presented continuously.

The observations are interpreted as demonstrating a specific reaction in photosynthesis associated with chlorophyll b (or other accessory pigment) and having time constants of the order of seconds.

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STUDIES OF REST PERIOD. II. NITROGEN AND PHOSPHORUS CHANGES IN EMBRYONIC ORGANS OF AFTER-RIPENING CHERRY SEED[1,2]

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Information on changes which occur during after-ripening is essential to an understanding of the mechanism of the rest period. In the first paper of this series (*7*) we reported that during after-ripening growth occurs in the embryonic axis of the cherry seed. This growth is accompanied by translocation of materials from cotyledons to axis, an increase in respiration rate of the axis, and an increase in the efficiency with which the respiratory enzyme system is utilized. These data suggest that the respiratory rise might be due to an increased supply of available phosphate or phosphate acceptors. The present work was performed to test this hypothesis. The observations are divided into two parts: A, measurement of changes in total nitrogen and phosphorus in the embryonic axis during after-ripening, and B, fractionation of total phosphate and measurement of the changes in quantities of these fractions during after-ripening. The results show that total phosphate concentration in the axis increases during after-ripening. This increase is not reflected uniformly in all fractions measured.

MATERIALS AND METHODS

Seeds of the sour cherry, *Prunus cerasus* L. var. Montmorency, were obtained and prepared by the methods previously described (*7*). The measurements of translocation of total nitrogen and total phosphorus were actually part of the experiment previously described; the sampling procedures and cell counts upon which figures 1 and 2 are based are detailed in that paper.

Total nitrogen was determined by titration following digestion according to the method of Boell and Shen (*3*) and recovery by diffusion according to a micromodification of the Conway method (*4*). Diffusion was carried out in paraffined vessels 13 mm in diameter and 13 mm deep in which 18 μliter aliquots of the 6 N acid digest and 18 μliters of 8 N NaOH were mixed with a magnetic flea (*5*). A drop containing 18 μliters 0.04 N H_2SO_4 with bromcresol green indicator (*3*) was suspended from a glass plate covered with "Parafilm" and sealed in place with vaseline. The drop was prevented from shifting during manipulation by a circular depression formed in the parafilm with a dull No. 1 cork borer. After diffusion at room temperature for 70 minutes, the receiving acid was transferred with rinsing to micro tubes and titrated with 0.01 N NaOH delivered from a horizontal microburette and mixed with a magnetic flea (*5*). This modification of the Conway method is sensitive to 0.05 μg nitrogen per diffusion vessel.

Total phosphorus of leaf primordia was determined by the method of Bernhardt and Wreath (*2*). To facilitate pH adjustment, the digestion method was modified by evaporating the nitric-perchloric acid digestion mixture (N-PCA, 2.5 volumes nitric acid plus 1 volume 70 % perchloric acid) to a dry residue at 130° C. This residue was dissolved and converted to orthophosphate by heating with 1 N HCl; the pH was then adjusted to about 2.4 with NaOH. The unreduced phosphomolybdate yellow was intensified by adding acetone and measured at 430 mμ in a special microcuvette in the Beckman DU spectrophotometer. This method is sensitive to 0.025 μg phosphorus.

To determine both total nitrogen and total phosphorus in aliquots from a digest of an individual embryonic axis, a sealed tube digestion was adapted from Lowry (*6*). A minimum quantity of 12 N H_2SO_4 was used for digestion at approximately 500° C in sealed ampules. After digestion the ampules were opened under an air pressure of approximately 30 psi which was reduced slowly to prevent frothing. Aliquots of the solution were then analyzed for total nitrogen and total phosphorus as described above.

Following the fractionation procedure outlined below, phosphorus was analyzed by the method of Schaffer et al (*8*).

The experiments on fractionation of total phosphorus were performed with seeds from the 1958 crop. In Delaware cherries are normally picked at

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a stage in which the fruits do not readily separate from the stems; seeds from such fruits were used for all except the 1958 experiments. In 1958 the fruits were allowed to ripen on the trees to a stage which corresponded with that normally used for commercial canning and freezing of sour cherries. After air-drying, the dry stones were stored in sealed containers at 5° C until used.

Because of the small quantities of phosphorus in some of the fractions, three samples of five seeds each were analyzed at each time interval. The axis and leaf primordia were analyzed as a unit rather than separately as reported in the previous paper (7). Each sample of five axis-leaf primordia units was carried through the entire fractionation procedure separately. The data were then calculated on a unit basis to permit direct comparison with previous data.

To prepare the phosphorus fractions, the freshly dissected material was quickly frozen in dry ice in a mortar made from thick-walled pyrex capillary tubing (35 × 2 mm ID). The frozen material was powdered by pounding with a cold stainless steel pestle and hammer (9). The frozen tissue was rubbed into suspension in 50 μliters 10% perchloric acid (PCA) with a pyrex rod. After centrifuging at room temperature for 1 minute at 1,000 × G the supernatant was transferred to a 6 × 45 mm micro tube. The precipitate was washed twice with 50 μliter aliquots of 5% PCA and the washings were added to the supernatant. The residue was dried, digested, and analyzed to provide a measurement of acid-insoluble phosphorus. To the combined supernatant approximately one milligram of specially purified Norite A carbon was added, the solution was stirred, and the carbon was removed by centrifugation. This carbon precipitate was then washed three times with 100 μliters of glass distilled water and the supernatants were combined. The total solution was then divided into two equal aliquots, one of which was analyzed directly for inorganic orthophosphate and the other dried, digested with N-PCA mixture, and analyzed to give the acid soluble non-nucleotide phosphorus residue.

The nucleotides adsorbed on the charcoal were hydrolyzed in 75 μliters 1.0 N HCl at 100° C for 12 minutes; the time for hydrolyzing high-energy phosphorus on carbon was found to be slightly longer than in solution. The carbon was washed once with 100 μliters water and the combined supernatants analyzed to give high-energy nucleotide phosphorus. The remaining adsorbed nucleotides containing the low-energy nucleotide phosphorus were determined by complete digestion on the carbon in 25 μliters N-PCA, followed by analysis in the presence of partially-digested carbon. Since the analysis required octanol extraction, this carbon did not interfere, but required special purification to remove contaminating phosphates.

This fractionation procedure produced five fractions, which, according to literature descriptions (2, 5, 9, 11) should have included the following types of compounds: High-energy nucleotide: adenosine di-and tri-phosphate. Low-energy nucleotide: adenosine monophosphate. Inorganic: inorganic orthophosphate. Acid soluble residue: chiefly pentose and hexose phosphates. Acid insoluble residue: chiefly nucleic acids and phosphoproteins.

Results

The data on total nitrogen (fig 1a, 1c) show an increase with after-ripening time at 5° C, but not at 25° C. Total phosphorus shows similar trends (fig 2a, 2c). These increases represent translocation of nitrogen and phosphorus from the reserves in the cotyledons to the growing cells of the axis. They are correlated with the breaking of rest at least to the degree that after-ripening at 5° C breaks rest. After-ripening at 25° C does not break rest but acts as a control to show changes which occur during moist storage.

Using the data on cell division previously published (7), the data (figs 1a, 1c, 2a, 2c) were recalculated to

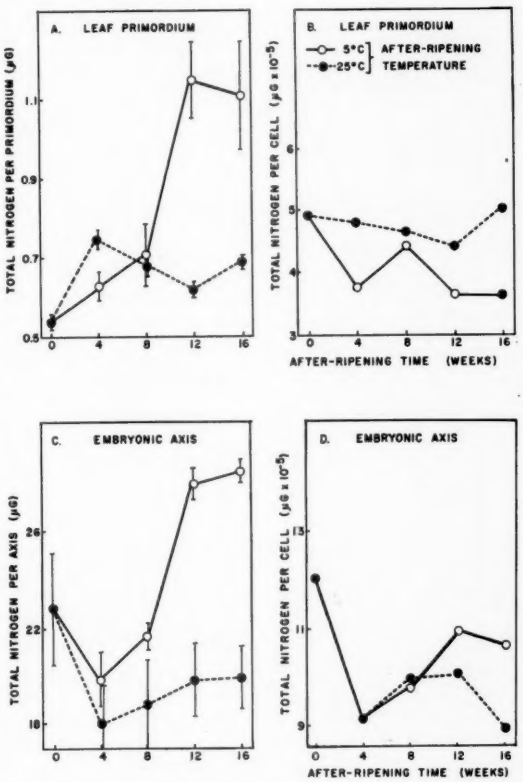

FIG. 1. The effect of after-ripening time and temperature on the total nitrogen content of cherry embryonic leaf primordia and axes. The vertical lines represent ± the standard error of the mean.

show the changes in quantity of nitrogen and phosphorus per cell (figs 1b, 1d, 2b, 2d). In the case of total nitrogen, the rate of translocation almost exactly parallels the rate of cell division, resulting in a constant amount of nitrogen per cell. However, the amount of phosphorus per cell increases with after-ripening at 5° C. Since this increase occurs in cells whose average nitrogen content remains constant during cell multiplication, this may be interpreted as a real increase in total phosphorus, i.e., phosphorus concentration per unit protoplasm.

Between 12 and 16 weeks after-ripening at 25° C there is a sharp increase in phosphorus per leaf primordium cell (fig 2b). This is the result of a relatively small increase in total phosphorus per leaf primordium (fig 2a) coupled with a relatively stable cell number. It was noted that at this time a number of seeds from this particular seed crop were actually capable of limited growth; this abnormally high concentration of total phosphorus may reflect that growth potentiality.

In the previous paper (7) data were presented to show that after-ripening at 5° C results in increased efficiency in utilization of the respiratory enzymes present. Such increased efficiency might be the result of increased availability of phosphorus or phosphate acceptors in the respiring cells. Since the data (fig 2) show such an increase in phosphorus concentration, it seemed desirable to determine whether this increase represents a general increase in the level of phosphate compounds, or whether it is the result of an increase in a particular class of compounds. Therefore, data were obtained in an experiment performed as nearly as possible under conditions identical to those of the experiment described in the previous paper and illustrated in figures 1 and 2 above.

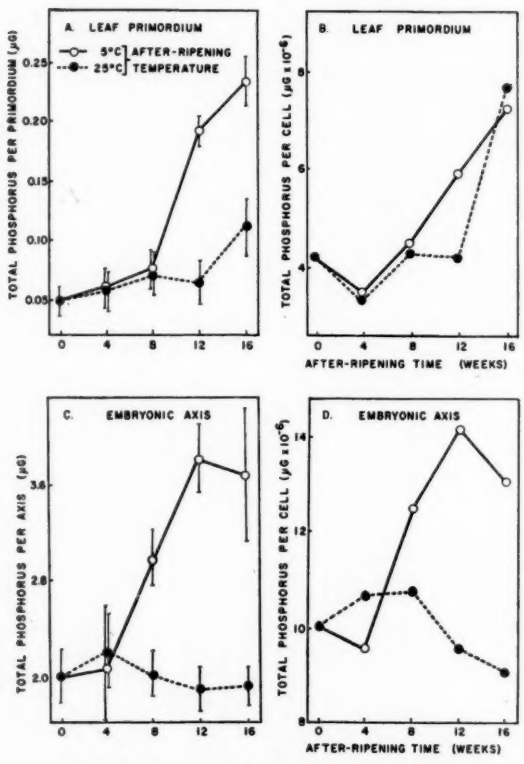

FIG. 2. Effect of after-ripening time and temperature on total phosphorus content of cherry embryonic leaf primordia and axes. The vertical lines represent ± the standard error of the mean.

The data in figures 3a to e show the comparison of phosphorus fractions from seeds after-ripened at 5° and at 25° C. In the nucleotide high- and low-energy phosphorus fractions, as well as the acid insoluble residue, the data are much as would be expected. These fractions increase with after-ripening at 5° C but not at 25° C; the quantities are higher in material after-ripened at 5° C than at 25° C. However, the fractions containing inorganic phosphorus (fig 3d) and acid-soluble phosphorus (fig 3e) are distinctly different. The general increase with after-ripening time is not apparent. More important, the quantities are higher, in some cases very much higher, in material from seeds after-ripened at 25° C than at 5° C. This difference is completely reversed from that of any quantity previously related to after-ripening.

In figure 3, the values of the ordinates vary with the different fractions. In the non-after-ripened axis, the bulk of the total phosphorus appears in the acid-soluble and insoluble fractions (55% and 27%), while the nucleotide and inorganic fractions are relatively small. After-ripening at 5° C results in a decrease in the percentage of acid-soluble fraction and some increase in the relative amount of the low-energy nucleotide fraction. Otherwise, the relationship between the fractions remains approximately constant. However, with 25° C after-ripening, the acid-soluble residue remains the largest fraction, and the inorganic phosphate fraction increases in amount at the expense of the acid-insoluble fraction.

Because of the time-consuming nature of the analyses, it was not possible to perform all of the experiments with seeds from the same crop. The experiments in the previous paper (7) and in figures 1 and 2 of this paper were performed with seeds of the 1957 crop; experiments on phosphate fractionation utilized seeds from the 1958 crop. By totaling the amounts of phosphorus in the various fractions, it is possible to directly compare the 1957 and 1958 crops. This comparison is shown in figure 4. The shape of the curve is similar in the two cases, but the magnitude of total phosphorus is very different, being almost twice as high in the 1957 as 1958 crops. Some of the data obtained suggest that the rates of after-ripening and growth were slightly different between the two crops, but in the absence of more direct and careful comparison, it is difficult to determine whether or not significant differences did exist.

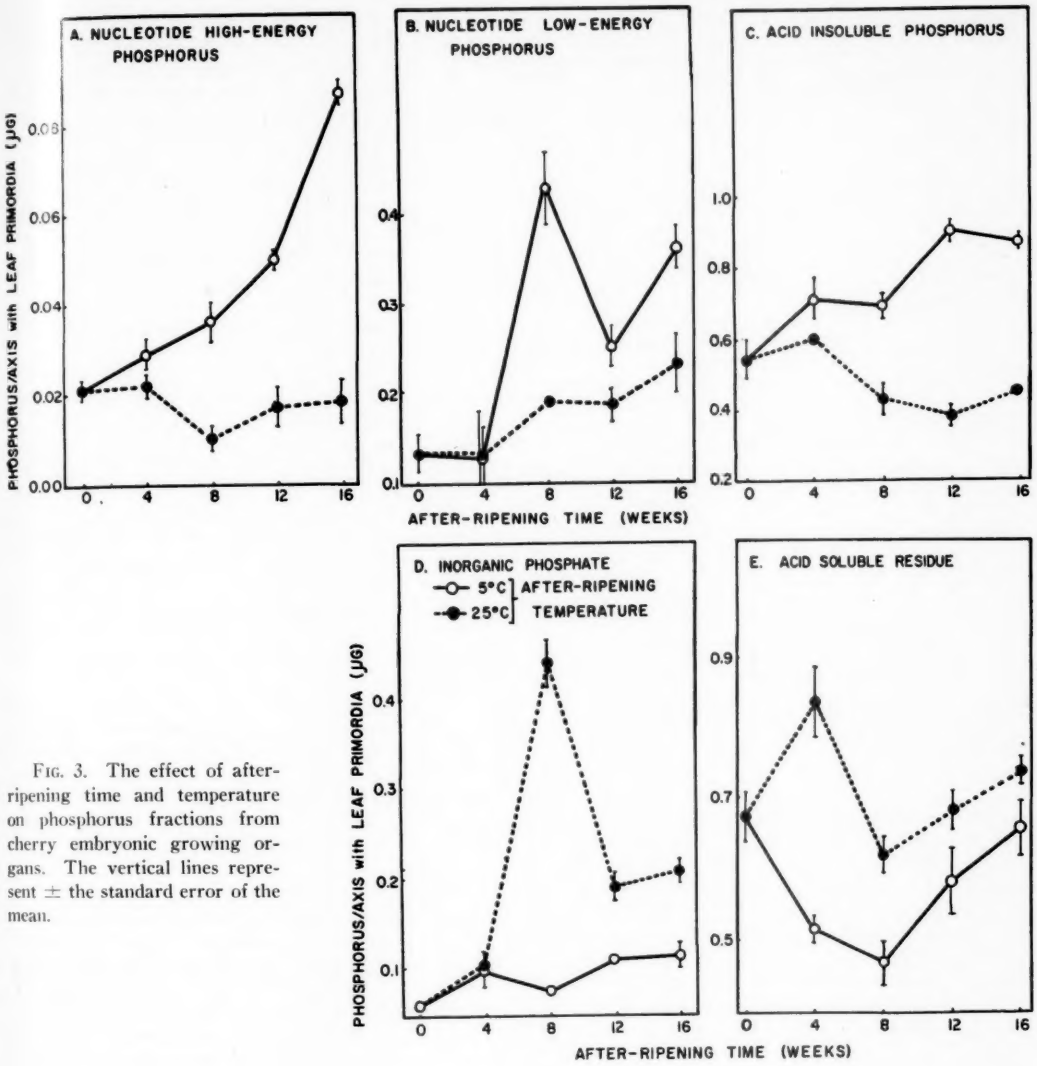

FIG. 3. The effect of after-ripening time and temperature on phosphorus fractions from cherry embryonic growing organs. The vertical lines represent ± the standard error of the mean.

DISCUSSION

The total amount of material in the embryonic axis of the cherry seed is almost insignificant in comparison with the total material in the storage organs. For example, the dry weight and total phosphorus of the axis plus leaf primordia is only about 0.7 % of the total in the seed. Because of the errors inherent in analytical methods, it is not possible to balance the gains in the growing organs against losses in the storage organs. Therefore, the gains in the embryonic axis have been assumed to be the result of translocation from the cotyledonary reserves. Although these gains are expressed on a per cell basis, this does not imply that the cells in the embryonic growing regions are structurally or physiologically equivalent. The obvious specialization of cells within the growing organs has not been included in the present study.

The observation that a close correlation exists between nitrogen translocation and cell division suggests that the growth occurring during after-ripening is primarily the result of cell division rather than cell enlargement. There is one obstacle to the interpretation of these data, however. In the seed, storage is not restricted to the storage organs. A certain amount of storage also occurs within the potentially growing cells of the embryonic axis. This can be seen in a gross way by noting the change from an

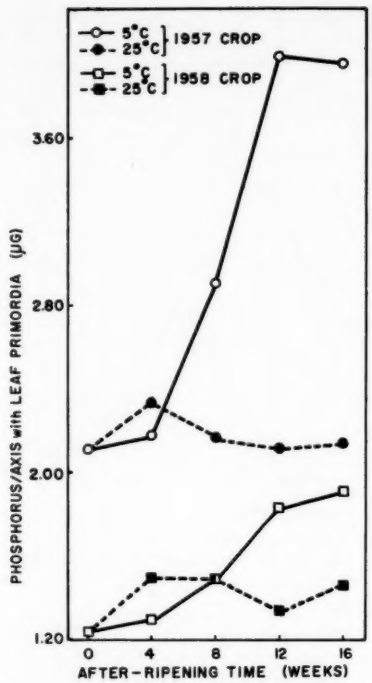

FIG. 4. Comparison of the total phosphorus in cherry embryonic organs of the 1957 and 1958 crops.

opaque to a translucent condition as the embryonic organs begin active growth. Microscopic examination shows the disappearance of starch grains and fat droplets at this time; these reserves are apparently the first used during active growth. Gross chemical analysis cannot distinguish between constituents of the cell, for example proteins, which are present as storage materials, and those which are present as protoplasmic components. Other types of measurement must be developed to measure the shift from storage to active compounds within any particular class of compounds.

The changes in phosphate compounds are closely correlated with the changes in respiratory activity previously noted (7). During after-ripening at 5° C the efficiency of utilization of the respiratory system, as measured by the respiratory increase in the presence of 2,4-dinitrophenol, increases. During 25° C after-ripening respiratory efficiency remains constant or declines. These results might be explained on the basis of an increasing supply of phosphate or phosphate acceptors during 5° C after-ripening, and not at 25° C. The data presented in this paper are consistent with this hypothesis. During after-ripening sufficient to break rest, phosphate is translocated to the growing organs; this translocation is in excess of the rate of growth, and so the effective phosphate concentration increases.

The present data go beyond the relation of total phosphate concentration to respiration and the breaking of rest. The accumulation of phosphate compounds in embryonic organs after-ripened at 5° C shows a pattern consistent with the movement of inorganic phosphate into the metabolic system through such intermediate compounds as sugar phosphates and nucleotides into more stable protoplasmic components such as nucleic acids, i.e., a normal synthetic system is operative. In seeds held moist at 25° C and not progressing toward the growing condition, the data suggest a breakdown of the more stable types of compounds and the accumulation of inorganic phosphate. The normal pathways of metabolism and synthesis are not functioning for growth.

In the absence of actual measurements on phosphate turnover, the above considerations remain suggestive and speculative. They might, however, be interpreted as suggesting that the rest period is associated with a metabolic block in the phosphorylation system between the initial phosphorylation of respiratory intermediates and the synthesis of end products. However, they might equally well be interpreted as being the result of a metabolic block elsewhere in the synthetic system which merely is reflected in the accumulation of metabolic intermediates.

It should be emphasized that there is a further problem of interpreting these results in relation to the rest period. It has been shown that in the cherry embryonic growing organs the rest period does not constitute an absolute block to growth; slow growth in the form of cell divisions and accumulation of metabolic constituents does occur even in the earliest stages of after-ripening. It is possible that the changes noted here are associated primarily with this slow growth and only secondarily with the breaking of rest.

The difference between the two years' cherry crops in content of total phosphate raises a question as to the normal differences of distribution of elements and compounds within the seed, and how these may be related to environmental conditions and to physiological behavior of the seed. Von Abrams and Hand (10) have presented data for Rosa indicating a relationship between pre-harvest environment and dormancy. The present data suggest this type of phenomenon.

SUMMARY

During after-ripening at 5° C, nitrogen and phosphorus are translocated from the storage to the potentially growing organs of the cherry seed. The rate of translocation of nitrogen is equal to the rate of cell division, so the nitrogen concentration per cell remains constant. However, the rate of translocation of phosphorus is in excess of cell division and the concentration of phosphorus in the cells increases. Fractionation experiments suggest that the translocated phosphorus moves through normal synthetic pathways into all phosphate compounds in the cells. However, in seeds held moist at 25° C and not pro-

gressing toward the growing condition, phosphorus tends to shift from the normal compounds such as nucleic acids and accumulate as inorganic phosphate. These data are in agreement with previous observations on respiratory rates and pathways. Although these data suggest that the rest period may be associated with a block in the phosphate metabolism of the cells, this interpretation cannot be fully substantiated.

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STUDIES OF REST PERIOD. III. RESPIRATORY CHANGES IN LEAF PRIMORDIA OF MAPLE BUDS DURING CHILLING [1, 2]

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Respiration is one of the physiological processes frequently measured in relation to the rest period. In general, respiration increases slowly with the shift from the resting to the potentially growing condition, and then very rapidly with the beginning of active growth. Obviously there is a limitation to respiration during early rest; this limitation is removed as exposure to low temperature removes the rest period block.

Few data are available in which the changes occurring in the growing organs can be separated from changes in the storage or protective organs, and in which an analysis of limiting factors has been attempted. Pollock (5) investigated respiratory changes in bud scales and growing regions of *Acer platanoides* buds, and concluded that respiration is limited by oxygen diffusion through the thick, living, respiring layer of bud scales. Working with *Pinus strobus* buds in which the rest period was already broken, Kozlowski and Gentile (3) noted a similar limitation to oxygen diffusion imposed by the nonliving scales. In addition, they noted a relationship between oxygen uptake and water content of the tissues, with a deficiency of water acting to limit oxygen uptake.

In the case of seeds, work on the respiration of cherry embryonic leaf primordia showed a respiratory limitation possibly associated with phosphate metabolism. Using 2,4-dinitrophenol, it was shown (6) that the respiratory efficiency of such leaf primordia changes with after-ripening; respiratory enzyme utilization seems to become more complete as the rest period is broken.

A number of workers (1, 2) have considered that the mechanism of rest in seeds (embryo dormancy) and buds is similar if not identical. It therefore seemed desirable to make a direct comparison between leaf primordia of a seed and leaf primordia of a bud to see whether or not similarities could be noted. Ac-

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cordingly, the effect of 2,4-dinitrophenol on the respiration of maple leaf primordia was determined after chilling outdoors or storage at room temperature. The techniques used for measurement were identical with those previously used (6) for cherry embryonic leaf primordia; the data are therefore directly comparable. The results show similarity between comparable physiological states in comparable organs at different stages in plant development and in unrelated species.

Materials and Methods

Two-year old 2 to 4-ft seedlings of *Acer saccharinum* L. were used. These trees were grown in the garden under normal environmental conditions, and pruned several times to produce a number of approximately uniform terminal buds on each tree. In November 1958, part of these trees were lifted, the roots were bagged, and the trees stored in a room with a temperature of 60° to 70° F and a low light intensity for 16 hours daily. The trees were covered with plastic and watered regularly to prevent desiccation. No buds opened during the experiments or for several months afterward; the trees remained in living and apparently healthy condition, although completely at rest. The remainder of the trees were left in the garden and exposed to normal winter temperatures to break the rest.

In November, at the time the trees were lifted, and in December and February, samples of buds were selected at random, brought into the laboratory daily, and dissected. Each bud contained three visible pairs of leaf primordia; the second pair was used since a single primordium from this pair respired at a rate easily measurable in a standard Cartesian diver. At the time of dissection, one primordium of each pair was fixed for cell counting; the respiration of the second primordium was measured and it was then dried overnight at 80° C and weighed. Calculations of respiration per cell and dry weight per cell were based on the fact that the opposite leaf primordia are well matched in size. The sample at each experimental time contained 15 to 20 buds.

Respiratory measurements were made at 25° C with techniques identical to those previously used (6). Because these leaf primordia contain chlorophyll, a green filter (7) was used on the Cartesian diver reading lamp to eliminate the possibility of photosynthesis. Cell counting and weighing techniques were also identical with those previously used.

Results

The sampling dates were selected to show trends during the breaking of rest, but to avoid the changes resulting from active growth later in the season. During the experimental period, there was no growth in the leaf primordia sampled. The average leaf primordium contained 42,600 cells and had a dry weight of 9 μg. Variations between the individual samples were not significant. The data presented in figure 2 were calculated from actual cell counts of matching leaf primordia, not from the statistical average of all individuals.

Figures 1 and 2 show that the normal rate of oxy-

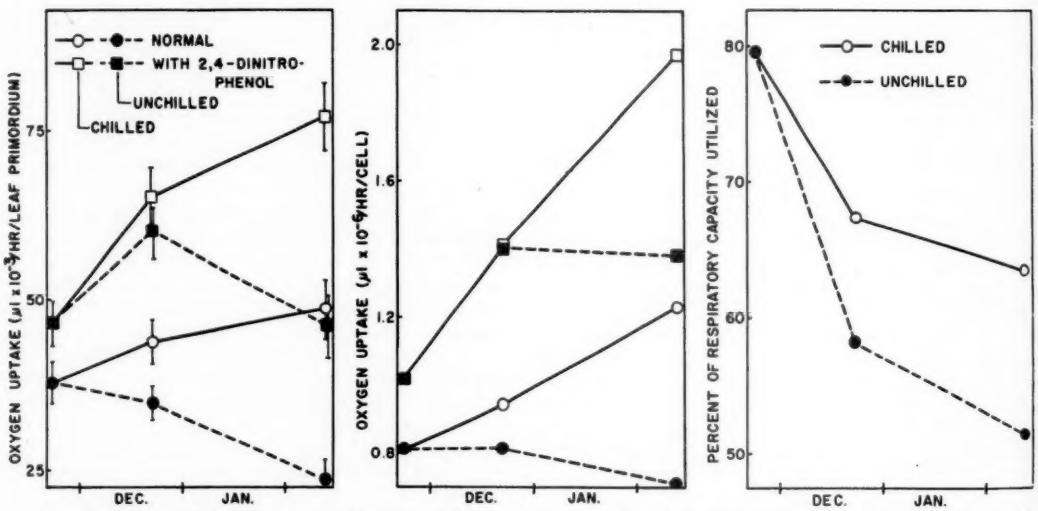

Fig. 1. (*left*). Respiration of maple leaf primordia from trees growing outdoors (chilled) and stored inside (unchilled). Measurements were made on individual leaf primordia in Cartesian divers. Each point is the average of leaf primordia from 15 to 20 buds; vertical lines are ± the standard error of the mean. Respiration measured at 25° C; 2,4-dinitrophenol concentration 3×10^{-5} M.

Fig. 2 (*center*). Data from figure 1 expressed on a per cell basis. Symbols as in figure 1.

Fig. 3 (*right*). The effect of after-ripening time and conditions on efficiency of utilization of respiratory capacity. Respiratory capacity is here defined as the maximum respiration rate in the presence of 2,4-dinitrophenol.

gen uptake rises slowly as a result of chilling, while that of buds from unchilled trees declines during the same period. Dinitrophenol increases the respiration rate: the percentage increase is higher in the warm-stored than in chilled buds. This difference increases with time. Thus, when the data are expressed as percentage of the total respiratory capacity utilized (fig 3), we find that the chilled primordia utilize the available respiratory capacity more fully than do unchilled primordia. However, in both cases the efficiency of utilization declines with increased duration of storage.

Discussion

When the present data are compared with those previously obtained for cherry embryonic leaf primordia (6), basic similarities are obvious. In both cases the respiratory rate rises with increased time of chilling, but remains constant or drops slightly with time in unchilled primordia.

Similarities in response to 2,4-dinitrophenol and the efficiency of respiratory utilization can also be seen. In both cases dinitrophenol increases respiratory rate; on a percentage basis this increase is higher in unchilled than in chilled primordia. Thus primordia from chilled seeds or buds are more efficient in utilization of available respiratory enzymes. The total respiratory capacity utilized ranges from approximately 50 to 80 % in the two cases examined. In cherry, the efficiency of chilled primordia is approximately 20 % higher than unchilled; in maple, this figure is 10 to 15 %. During early storage there is a transitory increase in response to 2,4-dinitrophenol which is similar in chilled and unchilled primordia, but does not persist in the absence of low temperature exposure.

The major difference noted between the two cases is that in cherry the degree of utilization of respiratory enzymes actually increases with chilling while in maple it decreases, but at a rate slower than in unchilled material.

Many more cases should be investigated to establish the general applicability of these results. However, the available data suggest that one of the effects of chilling is to maintain or increase the closeness of coupling between energy releasing and energy utilizing processes, i.e., respiration and synthesis. Conversely, the data might be interpreted as indicating that the rest period results from a block in the system of energy metabolism. As emphasized previously (4), however, the results could equally well be interpreted as secondary effects resulting from a primary block in a synthetic system.

Summary

Measurements of respiration rate and respiratory response to 2,4-dinitrophenol have been made at intervals during chilling and warm storage on leaf primordia from buds of *Acer saccharinum* trees. The respiration rate of buds from chilled trees rises as a result of previous low temperature exposure which breaks rest; respiration of unchilled buds drops during the same period. Dinitrophenol increases respiration rate; the increase becomes greater with length of storage but is always less in chilled than in unchilled buds. Thus the efficiency of utilization of the respiratory enzyme system always remains higher in organs approaching growth than in those in which the rest period is not broken. This situation is similar to that in embryonic leaf primordia from cherry seeds, and suggests that it may be a more general phenomenon.

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REMOVAL OF HEAVY METAL AND HALIDE CONTAMINATION FROM MACRONUTRIENT SALTS[1]

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In micronutrient deficiency experiments with plants the salts used to supply macronutrients must obviously be free of significant contamination. Hewitt (4), in reviewing the testing and purification of nutrient reagents, clearly pointed out that there is no guarantee that even the purest salts commercially available are sufficiently free of the known micronutrients, and that routine use of an appropriate purification procedure is amply justified by many examples of improvement gained in deficiency technique. Routine purification is further justified if it can be done more easily than analytical testing of reagents in search of the occasional highly pure batch.

While recrystallization has been extensively used as a general purification procedure, Hughes (5) and Hewitt (4) have pointed out that more effective processes for specific groups of micronutrients are needed. This view is supported by data on the effectiveness of recrystallization which we have included in this report.

We have applied a number of procedures with the aim of removing specific micronutrients. For example, micronutrient metals have been removed by adsorption on calcium phosphate (1), by coprecipitating with CuS and FeS (2, 4, 11) and by extracting with 8-quinolinol (4, 9), while chloride has been removed by adding $AgNO_3$ as well as by recrystallizing (2). Some of these processes have not been highly effective, and all are somewhat time-consuming. Accordingly we have developed two new procedures—evaporative treatment with HNO_3 to remove halides, and coprecipitation with $Mg(OH)_2$ to remove heavy metal cations. Both are exceedingly simple as well as effective.

METHODS

RADIOTRACER TECHNIQUES. In examining the $Mg(OH)_2$ process and in some of the recrystallization experiments, Co^{60} and Zn^{65} were used as tracers.

The stock solution of $Co^{60}Cl_2$ had a chemically unreactive "radio-colloid" fraction amounting to 5 % of the total activity. It could be converted completely to the reactive form by boiling with aqua regia. The non-colloid could also be separated from the radio-colloid by coprecipitation with $Mg(OH)_2$ or by adsorption as the anionic chloride complex on Dowex-1 anion resin (12). Aqua regia-treated Co^{60} was used in the experiments.

Crystals and precipitates were redissolved and 10 ml samples of solution were counted in beakers of matched size and shape, using a Tracerlab TGC-8 Geiger Müller tube with end-window of thickness greater than 500 mg/cm². Geometry, thickness and salt concentration were constant.

Purified specimens had low count-rates, and their counts were accumulated only sufficiently to give confidence limits within 30 to 50 % of the net count. (This was usually attained when net counts totalled 500 with a background count of the order of 5,000).

CHEMICAL PROCEDURES. Zinc was determined semiquantitatively by extracting large samples with dithizone into 2 ml of CCl_4 and matching the pink Zn-dithizone color visually against standards. The limit of ready detection was 0.1 μg of zinc. For quantitative measurements the method of Cowling and Miller (3) was used without dilution of the final CCl_4 extract.

Chloride was determined by potentiometric titration (7, 8) against 0.01 M or 0.001 M $AgNO_3$, to the apparent equivalence potential of a nominally chloride-free reference solution in the cell:

Ag-AgCl | test solution || KNO_3 sat. | quinhydrone.

Titrant was delivered from a Gilmont ultramicroburet and the endpoint detected with a Beckman model H2 pH-meter equipped with voltage stabilizer.

The presence of large amounts of other salt introduces difficulties in the titration of small amounts of chloride. Large errors arise from differences in junction potentials unless the reference solution has the same concentration of the same kind of salt as the unknown. Furthermore, the quantity which is actually measured by titration is the difference between the chloride content of the unknown and that of the reference, so that the reference must be of exceptional purity if the measurements are to have meaning. A series of specially purified nutrient salts was thus required for preparing reference solutions.

The relative purity with respect to chloride, of solutions otherwise alike, can be judged by the magnitude of the potential produced in the titration cell. The salts with lowest apparent chloride content which we could produce were those which had been treated three or four times in succession by wetting with concentrated HNO_3 and evaporating to dryness. The basis for this process is the well-known reaction:

$$NO_3^- + 3Cl^- + 4H^+ \rightarrow NOCl + Cl_2 + 2H_2O,$$

both chlorine-containing products being highly volatile. Increasing the number of HNO_3 treatments beyond four gave no further reduction in apparent chloride content; perhaps it is reasonable to assume that the salts then had no detectable chloride.

Good precision resulted when due attention was paid to preventing contamination, chiefly in the temporary abolition of hydrochloric acid from the laboratory. (Replicate analyses of 30 samples with chloride contents 1 to 4 μg gave a standard deviation of 0.06 μg).

[1] Received June 17, 1960.

RECRYSTALLIZATION. Experiments were run to provide quantitative information on the effectiveness of recrystallization in removing some of the micronutrients of special interest to us. Most of the reagent salts used in this study were so pure as to require deliberate contamination with $ZnCl_2$, in order that the recrystallized products would have chemically measurable amounts of zinc and chloride. This high degree of purity cannot be assumed for different lots of reagents. With cobalt, the concentrations which are conveniently measurable chemically are too high to be of interest to plant nutritionists. Hence Co^{60} was used, at rates of contamination of the order of 10 μg of cobalt per mole of salt.

Glassware was washed with M/2 trisodium hydrogen ethylene-diaminetetraacetate (Na_3EDTA), rinsed with glass-distilled water, then washed with 10 % reagent grade HNO_3, and rinsed again with glass-distilled water.

Preliminary experiments indicated that the practice of discarding a first fine crop of crystals, on the notion that it should scavenge contaminants from solution, achieves little more than the waste of the discarded salt.

The salt to be purified was dissolved in glass-distilled water at 90° C to make a saturated solution, which was then cooled slowly in an insulated box to 5° C, over a period of 3 days. The solution was seeded to induce crystallization when necessary. Unless large crystals grew, the solution was reheated to dissolve and the cooling repeated. Crystals were washed five or six times with glass-distilled water.

RESULTS OF RECRYSTALLIZATION EXPERIMENTS. Radiotracer experiments tend to overestimate the effectiveness of purification since they do not measure recontamination from atmospheric and other sources. Chemical analysis, on the other hand, includes all contributions from recontamination. The extent of recontamination can be checked by analyzing all the fractions from the recrystallization; the sum of their individual contents of zinc and of chloride will equal the initial contents if recontamination is insignificant. This was so in these experiments, so that the data obtained by chemical analysis is comparable with that of the tracer experiments.

Table I shows the concentrations of the test microelements remaining in crystals after crystallization, and also expresses this amount as a percentage of the original concentration. Recrystallization failed to lower the cobalt and zinc content of $MgSO_4$ to any useful extent, and sometimes even increased the zinc concentration slightly (e.g., 1st recrystallization, table I). This is not surprising. Zinc forms sulfates isomorphous with the magnesium sulfates, and the divalent ions of cobalt, copper, iron, and manganese form double sulfates with $MgSO_4$ (10). For the other salts and micronutrients, recrystallization achieved fair to good purification. There seems to be some tendency for the first recrystallization to be relatively more effective than the second. The wastefulness of the process is evident in the low yields of salt.

CO-PRECIPITATION WITH MAGNESIUM HYDROXIDE FOR REMOVING HEAVY METALS. Magnesium hydroxide offers advantages as a co-precipitating agent. It is insoluble, weakly basic, introduces no risk of toxicity to plants, and has a lattice structure like that of many other divalent metals (10). The following pro-

TABLE I

RECRYSTALLIZATION EXPERIMENTS

	KNO_3	$Ca(NO_3)_2$	$MgSO_4$	KH_2PO_4
Removal of chloride				
Conc μg/mole				
Initial	1,100	4,500	4,000	3,400
1st Recrystallization	160	870	400	420
2nd Recrystallization	...	235	50	190
% Residual	15	5	1	6
Salt yield %	60	25	7	55
Removal of zinc				
Conc μg/mole				
Initial	1,000	4,200	3,200	2,600
1st Recrystallization	85	880	3,800	410
2nd Recrystallization	...	230	2,200	100
% Residual	8	6	70	4
Salt yield %	60	25	7	55
Removal of Co^{60}				
μg Co/mole*				
Initial	5	...	20	15
1st Recrystallization	0.4	...	8	0.4
2nd Recrystallization	...	...	...	...
% Residual	8	...	40	2.5

* Approximate calculations from count rate, based on specific activity of initial Co^{60} preparation.

cedure has proved rapid and effective in removing cationic micronutrients from all salts except those of ammonium, where the low dissociation of NH_4OH prevents precipitation of $Mg(OH)_2$.

Glassware and filters were washed with EDTA and HNO_3 as described for recrystallization. Since EDTA and HNO_3 are themselves potent contaminants in the following procedure, water rinsing was thorough. Special attention was required in cleaning filters, but a filter once cleaned, was used a number of times until it clogged.

Ten millimoles of $MgSO_4$ or $Mg(NO_3)_2$ were added per liter of the concentrated (usually M/1) stock solution of macronutrient salt. Potassium hydroxide solution equivalent to this amount of magnesium was then added slowly and with vigorous stirring. Stirring was continued for at least half an hour; supersaturation rarely occurs but the precipitate sometimes forms slowly. The suspension was then filtered through a cleaned Whatman No. 12 paper.

The amount of $Mg(OH)_2$ precipitated may vary at least over the range 5 to 50 mmoles/liter without effect. Centrifugation may be substituted for filtration and is appropriate for small amounts of solution. The magnesium source and the KOH may be purified, if necessary, simply by adding a little of each to the other and separating off the precipitate.

Tests of $Mg(OH)_2$ Purification Procedure. Table II shows data of tracer experiments in which the order of contamination was 10 μg of the contaminant element per mole of salt. Comparison with table I shows improvement over recrystallization except with potassium phosphate.

Deliberately contaminated solutions of nutrient salts were put through the purification procedure and then subjected to a biological assay for micronutrients with tomato plants. The stock solutions, M/1 $Ca(NO_3)_2$, M/1 KNO_3, and M/2 $MgSO_4$, all from reagent-grade salts, were contaminated with 50 mmoles manganese per mole of salt, 20 of zinc, 5 of copper, 1 of molybdenum, 250 of boron, and 75 of chloride; rates which would supply more than ample of each micronutrient for plant growth. After two successive co-precipitations with $Mg(OH)_2$, dithizone tests indicated less than 5×10^{-3} μmoles of copper and zinc per mole of salt. Nutrient solutions were prepared according to Johnson, Stout, Brover, and Carlton (6), using the purified salts, with recrystallized KH_2PO_4[2] and pyrex-redistilled water. Four tomato plants were grown in each 4-liter pyrex beaker of nutrient.

Table II

Tracer Experiments With $Mg(OH)_2$ Procedure

Salt solution	Contaminant	% of Contaminant after single treatment
KNO_3 M/1	Co–Co^{60}	0.8–1.4
$Ca(NO_3)_2$ M/1	"	1.0–1.5
$MgSO_4$ M/2	"	5.0–6.0
K_2HPO_4 M/1	"	5.0
KNO_3 M/1	Zn–Zn^{65}	2.0
$MgSO_4$ M/2	"	1.0

Cultures to which manganese, zinc, or copper were not added produced severe symptoms of deficiency of the appropriate nutrient (13), with negligible growth and widespread necrosis in the manganese and zinc deficiencies. As expected, no responses to chloride, boron, or molybdenum occurred, indicating ineffectiveness of $Mg(OH)_2$ precipitation in removal of anions. (Contamination from Pyrex supplies a small amount of boron, but insufficient to prevent tomato plants from developing severe deficiency if the macronutrient salts are boron-free.)

Besides manganese, copper, zinc, and cobalt—the elements tested here—there is little doubt that ferric and ferrous iron would also be removed, as well as most other metals whose bases are substantially less soluble than $Mg(OH)_2$.

Cobalt and other contaminants may possibly occur in colloidal form, similar to the "radio-colloid" of our $Co^{60}Cl_2$ solution. Treatment of the salts with aqua regia as a preliminary to purification, converting all the contaminant to a form susceptible to removal, might be desirable for critical micronutrient experiments.

Nitric Acid Evaporation Treatment for Removing Halides. The successful use of HNO_3 evaporation in preparing low-chloride reference salts for potentiometric titration suggested its use for large-scale purification. The dry salt to be purified was weighed and placed in a large beaker or Erlenmeyer flask, to form a layer not more than 15 mm deep. One or two boiling chips and enough concentrated reagent HNO_3 to submerge most of the salt were added. The mixture was gently boiled on a hotplate or sandbath, almost to dryness. This treatment was repeated two or three times, and the salt was finally taken to complete dryness, dissolved, and made to volume with glass-distilled water.

Phosphate salts tend to form pyrophosphates when strongly heated with acid and in the final evaporation were heated gently on a steambath.

Tests of Nitric Acid Procedure. The experience already described in connection with reference solutions for chloride titration constitutes the best laboratory test available.

The following greenhouse experiment was undertaken to test whether or not chloride-deficiency could be produced on batches of $MgSO_4$, KNO_3, and $Ca(NO_3)_2$, which had been digested with aqua regia as a first step in preparing for experiments with cobalt in legumes. The aqua regia introduced about 2 moles of chloride per mole of macronutrient. Three evaporative treatments with concentrated HNO_3 followed

[2] Reasons for use of recrystallized KH_2PO_4 are given in the discussion.

TABLE III

MICRONUTRIENT PURIFICATION PROCEDURES

METHOD	PURPOSE	APPLICABILITY
Evaporative treatment with HNO_3.	Removal of halides and boron	All commonly used macronutrient salts except those of ammonium
Co-precipitation with $Mg(OH)_2$.	Removal of heavy metal cations	All commonly used macronutrient salts except those of ammonium
Co-precipitation with CuS under acid conditions (4).	Removal of molybdenum	All commonly used macronutrient salts
Recrystallization or other appropriate procedure.	Removal of halides, boron, and heavy metals	Ammonium salts; KH_2PO_4

the aqua regia treatment. The salts were then dissolved and the solutions submitted to $Mg(OH)_2$ co-precipitation.

Nutrient solutions were prepared as previously, using the above salts, recrystallized KH_2PO_4, and glass-distilled water. Four tomato plants were grown in each 4-liter culture. Besides controls with a full supply of micronutrients added, the differential treatments consisted of separate omission of each of the micronutrients chloride, boron, molybdenum, manganese, copper, zinc, and cobalt.

Control plants grew well, indicating no toxic effect of treatment with aqua regia. Pronounced symptoms of deficiency (13), including reduction of growth, developed for all the micronutrients except cobalt, which has not yet been shown essential to non-leguminous plants. This indicates that $Mg(OH)_2$ treatment need not add significant contamination of anionic micronutrients. More important, the appearance of chloride deficiency after heavy initial contamination with chloride indicates that evaporative treatment with HNO_3 is effective.

Although no tests have been carried out, HNO_3 treatment as described here might well remove fluoride, bromide, iodide, and borate, as well as chloride.

DISCUSSION

We chose, for the greenhouse experiments reported here, to retain recrystallization for purifying potassium phosphate. Reasons of convenience determined this choice and it was a fairly open one in any case. When applied to phosphates the new procedures are at least as effective as recrystallization, but involve small practical difficulties. Thus treatment with HNO_3 tends to form pyrophosphate, and the $Mg(OH)_2$ treatment produces K_2HPO_4, which then has to be neutralized with purified acid. On the other hand KH_2PO_4 recrystallizes especially easily and efficiently.

The HNO_3 and $Mg(OH)_2$ processes of course do not comprise a completely general purification scheme; they do not remove molybdenum, nor are they applicable to ammonium salts.

An approach to a comprehensive scheme (as illustrated in table III) would consist of a repertory of methods, each chosen for its suitability to a particular purpose.

SUMMARY

Two simple procedures have been developed for removing halides and heavy metal cations from the salts used to supply macronutrients in sand and solution cultures. The heavy cations are co-precipitated with magnesium hydroxide, and the halides are removed by wetting the dry salt with concentrated nitric acid and evaporating it to dryness. Chemical and biological assays have established the effectiveness of these procedures.

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INVESTIGATION OF HYOSCYAMUS NIGER L., A LONG-DAY PLANT FOR ENDODIURNAL PERIODICITY IN FLOWERING RESPONSE[1]

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Rhythmical phenomena in plants had been observed long before the discovery of photoperiodism by Garner and Allard (5) in 1919. In 1936, Bünning (2) proposed an hypothesis, based largely on observations of leaf movements, that photoperiodic induction was under the control of a rhythmical process. In order to trigger floral initiation in diurnal cycles, a minimum duration of light or darkness (depending upon the variety of plant) must elapse; thus, a sense of timing, or a clock, is associated with the induction process. Because rhythms, by their nature, might function as clocks, and because rhythms are not foreign to higher plants, the Bünning hypothesis has remained as reasonable a postulation as any which have been advanced, despite a dearth of substantial supporting evidence.

For a detailed account of rhythms in (plant) photoperiodism, the reader is referred to Bünning (3), and Hamner (6).

In 1957, Blaney and Hamner (1) reported the results of experiments in which short-day Biloxi soybean plants were exposed to several cycles which consisted of two phases: A, an 8-hour photoperiod, and B, a dark period whose duration was different in each of the several treatments. Maximal flowering responses were observed in cycles of 24 hours' and 48 to 52 hours' duration, while minimal responses were shown in cycles of 32 to 36 hours' and 56 hours' duration. Later, Nanda and Hamner (8) showed that another maximal response occurred in cycles of 72 hours' duration.

This report concerns efforts directed at determining periodicity in the flowering response of the long-day plant *Hyoscyamus niger*.

MATERIALS AND METHODS

Hyoscyamus niger L. was selected because it is a long-day plant which has a well-defined photoperiodic behavior (7) and especially because it has a potential for a rhythmical floral response (4).

Seeds of the annual variety of Hyoscyamus, obtained from our colleague, Dr. Anton Lang, were sown in aluminum boxes containing washed sand. The seedlings were periodically watered from the bottom while being maintained under short-day conditions in the greenhouse and, after several leaves had formed they were transplanted into plastic cups, one plant per cup. Sunlight intensity usually reached 7,500 ft-c or more. Night temperatures ranged from 10° to 17° C and the day temperatures from 13° to 43° C. Low humidities were avoided through the use of humidifiers and general wetting-down of the greenhouse during watering. In spite of carbon filtration of the incoming air (to the greenhouse), from time to time, the plants showed acute smog damage. When smog-induced lesions appeared, plants were removed to smog-free control rooms, where they were kept under short days for about two weeks prior to treatment.

All experiments were conducted in facilities at the Plant Physiology Building, University of California at Los Angeles. Plants were treated in temperature-controlled walk-in refrigerator rooms, lightproof unrefrigerated rooms, or in open areas. In the latter two places, the temperature varied slightly with the season of the year, but was fairly constant within the relatively short period of experimental treatment. Artificial illumination was supplied by cool-white fluorescent lamps. Two or more 100 w incandescent lamps were associated with each group of 12 fluorescent lamps. Light intensity is noted in each experiment.

The strength of induction, or flowering response, was measured by the number of days to appearance of elongation. A daily or bi-daily inspection of the rosette was made to determine the average number of days from the beginning of treatment to the appearance of the floral stalk.

EXPERIMENTAL

EXPERIMENT I. The Bünning hypothesis postulates that the rhythms of short-day plants and long-day plants are 12 hours out of phase with one another. Blaney and Hamner (1) and Nanda and Hamner (8) observed maximum floral responses in cycles of 24 and 48 hours' duration and a minimum in a cycle of 36 hours' duration in the short-day plant Biloxi soybean. Thus, in the long-day plant Hyoscyamus, a cycle of 36 hours' duration might be expected to produce a maximum response in comparison with a cycle of 24 hours' duration. Experiment I was designed to test this hypothesis.

Since Hyoscyamus requires a minimum duration photoperiod of 10.75 hours at 22° C (7) for the induction of flowering in 24-hour cycles, a severe test of the hypothesis was proposed in limiting the photoperiods of 36-hour cycles to a normally subthreshold photoperiod of 9 hours. Continuous light treatments, which evoke a stronger (earlier) flowering response than long-day 24-hour light-dark cycles, were used for comparison with the flowering response caused by 36-hour cycles.

Twenty plants of similar size and aspect were selected and then subdivided randomly into five groups

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of four plants each. The five groups were placed in a controlled-temperature room. Due to mechanical failure, the temperature during the first 24 hours was 31° C, but thereafter was 23° to 24½° C. Light intensity was about 750 ft-c at the leaf surface.

The five groups were treated separately as follows:

I. In greenhouse; under short-day conditions consisting of 8 hours' light and 16 hours' darkness.

II. In controlled-temperature room; under continuous light until appearance of elongation, or end of experiment.

III. In controlled-temperature room, and given 81 hours of continuous light, then returned to short-day greenhouse conditions.

IV. In controlled-temperature room and given nine cycles of treatment; each cycle consisted of 9 hours' light and 27 hours' darkness.

V. Given the same treatment as in (4) above except that treatment was continuous until appearance of elongation or end of experiment.

The results are shown in table I. The data in treatments No. 4 and 5 indicate that the flowering response was greater in a 36-hour cycle than in a 24-hour cycle, since the 81 hours' continuous light in treatment No. 3 would evoke the same, or an earlier, flowering response than long-day 24-hour cycles. The response in treatments No. 4 and 5 compared favorably with the response of a continuous light treatment No. 2. All the plants in treatment No. 3 were vegetative at the time of dissection. Thus, comparing treatments No. 3 and 4, the data indicate that darkness, per se, did not inhibit, but promoted flowering.

EXPERIMENT II. A number of treatments were exposed to light-dark cycles ranging from 18 hours' through 72 hours' duration. As in Experiment I, the photoperiod in each treatment was initially 9 hours long. For the first 669 hours of the experiment, the 9-hour light period was maintained; but, because of the sluggish response of treatments receiving cycles in excess of 18 hours' duration, the length of the light periods was increased in all treatments to 12 hours' duration. All treatments were terminated 797 hours after the start of the experiment, except the following, which were terminated at the times stated:

I. The 42-hour-cycle treatment at 872 hours after the start of the experiment.

II. The 45-hour-cycle treatment at 920 hours.

III. The 48-hour-cycle treatment at 991 hours.

IV. The 51-hour through 72-hour-cycle treatments at 1,055 hours.

One-hundred seventy-six Hyoscyamus plants of similar size and aspect, 18 weeks old, were divided randomly into groups of eight plants each. Light intensity was 890 to 950 ft-c. Temperature during the light period was 21° to 23° C; during the dark period, 20 to 22° C.

Figure 1 shows that a flowering response was obtained in cycles up to and including 18 hours' duration, but in cycles of 24 and 30 hours' duration, flowering was completely suppressed. Flowering reappeared in treatments receiving cycles of 33 hours' duration or more. A second wave of inhibition was not observed in longer cycles.

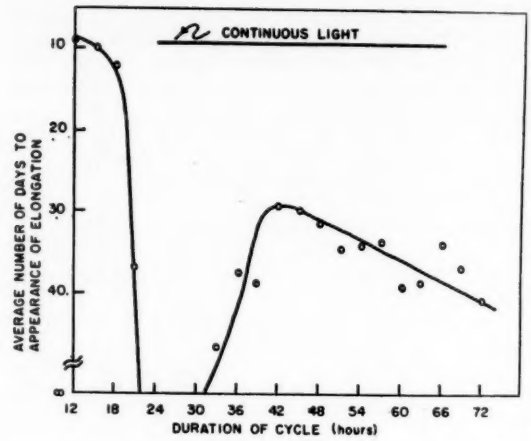
We defined vigorous Hyoscyamus plants as those having compact rosettes, dark green leaves, petioles about two centimeters in length, and an average mature leaf width/length ratio of about 0.65. Low vigor was defined as being characterized by distended rosettes, yellow-green green leaves, petioles of about four centimeters in length, and an average mature leaf width/length ratio significantly less than 0.65. Groups which received cycles of 30 hours' duration or less were rated as vigorous; groups receiving cycles in excess of 30 hours' duration exhibited a gradual decline in vigor—the longer the cycle, the lower the vigor.

TABLE I

FLOWERING RESPONSE OF HYOSCYAMUS NIGER ANN. TO 36-HOUR LIGHT-DARK CYCLES (EXPT I)

TREATMENT	DAYS FROM BEGINNING OF TREATMENT TO APPEARANCE OF ELONGATION									CONDITION OF APICAL MERISTEM*
	9	10	11	12	13	14	15	16	17	
(1) Continuous cycles of 8 hours' light and 16 hours' dark in greenhouse	...	...	...	(No response)			...	...	...	All vegetative
(2) Continuous light until end of experiment	X**	X	X	...	...	X	...	...	...	All flowering
(3) 81 hours' continuous light, then short days	...	...	...	(No response)			...	...	...	All vegetative***
(4) 9 Cycles of 9 hours' light and 27 hours' dark; then short days	...	...	...	...	...	XX	...	...	XX	All flowering
(5) Continuous cycles of 9 hours' light and 27 hours' dark until end of experiment	...	...	...	X	...	XXX	...	...	...	All flowering

*18 days after beginning of treatment.
** One X = one plant.
*** Meristems were slightly domed.



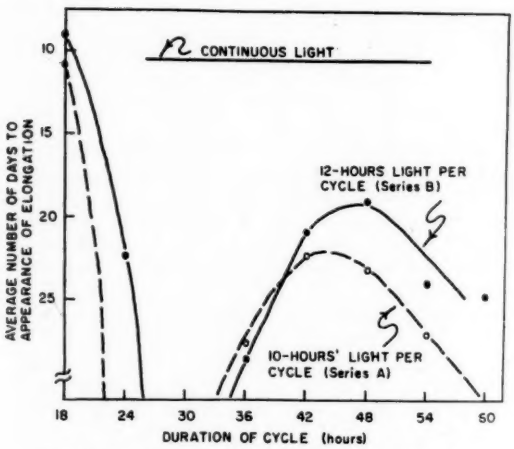


FIG. 1 (*top*). Experiment II. The flowering response of *Hyoscyamus niger* when exposed to cycles of various duration (9-hour photoperiod) as measured by the *average number of days to appearance of elongation.* Ordinate value at abscissa represents *no elongation* at the time of dissection.

FIG. 2 (*bottom*). Experiment III. The flowering response of *Hyoscyamus niger* when exposed to cycles of various duration (Series A: 10-hour photoperiod; Series B: 12-hour photoperiod), as measured by the *average number of days to appearance of elongation.* Ordinate value at abscissa represents *no elongation* at the time of dissection.

It would seem that a variation in temperature between light and dark periods would not enhance vigor appreciably because the most vigorous plants received the same duration of exposure to constant temperature as did some of those showing reduced vigor. The amount of light available for photosynthesis would seem to be the factor responsible for reduced vigor. It is possible that the expression of a periodicity in the flowering response in longer cycles may be obscured by a loss of vigor.

EXPERIMENT III. Because of the sluggish response experienced in Experiment II, two series of light-dark cycles were employed: In Series A, each cycle was given a 10-hour photoperiod; in Series B, a 12-hour photoperiod. One-hundred thirty-six Hyoscyamus plants of similar size and aspect, about 14-weeks old, were randomly divided into groups of eight plants each. Light intensity was 890 to 950 ft-c. Temperature during the light period was 21° to 23° C; during the dark period, 20° to 22° C. All treatments were continued for about 735 hours.

The results of Experiment III are presented in table III. The data for *the number of days to the appearance of elongation* are presented in figure 2. The response in both series was similar to that experienced in Experiment II; that is, a suppression of flowering in cycle lengths of 24 and 30 hours' duration followed by a resurgence of flowering in longer cycles. Series B (12-hour photoperiod) showed a stronger resurgence than Series A (10-hour photoperiod).

TABLE II

FLOWERING RESPONSE OF HYOSCYAMUS NIGER ANN. TO CYCLES OF SEVERAL DURATIONS (EXPT II)

DURATION OF CYCLE, HR	AVERAGE DAYS TO APPEARANCE OF ELONGATION
Continuous light	9.1 ± 1.0
12	8.8 ± 1.1
15	9.5 ± 0.5
18	11.0 ± 0.0
21	37.0 ± 2.6
24	No response
27	No response
30	No response
33	46.8*
36	38.3 ± 6.6**
39	39.8 ± 3.7**
42	28.1 ± 2.3
45	28.3 ± 2.2
48	31.5 ± 1.0
51	35.3 ± 3.5
54	34.0 ± 1.0
57	34.4 ± 1.8
60	40.8 ± 5.6
63	39.0 ± 1.8
66	36.6 ± 1.5***
69	38.6 ± 4.6
72	42.0†

Initially, light-dark cycles in each treatment contained 9-hour photoperiods; cf. text for changes. Eight plants responded in each treatment, except as noted. Standard deviations shown.

* One plant elongated; seven did not.
** Seven plants elongated; one did not.
*** Seven plants elongated; one died during treatment.
† Two plants elongated; six died during treatment.

Discussion

It has been determined (1, 8) that two waves of inhibition occurred in Biloxi soybean: one was maximal in cycles of 32 and 36 hours' duration and the other in cycles of 56 hours' duration. In the long-day plant Hyoscyamus, one wave of inhibition appeared in cycles about 24 to 30 hours long, or 6 to 12 hours before the first wave of inhibition in the short-day plant Biloxi soybean. It is possible that a similar rhythmic pattern might be present in Hyoscyamus but, as discussed earlier, its expression may have been suppressed by the lowered vigor of the groups which received longer duration cycles. At any rate, it cannot be concluded that a rhythm is indicated by our data.

However, the occurrence of the pattern of inhibition reported here reveals a new aspect of the photoperiodic induction process. In the long-day *Hyoscyamus niger*, it seems clear that a short day inhibits flowering not because the photoperiod is too short but because the duration of the light-dark cycle is unfavorable for flower induction. Therefore, it is inaccurate to simply state that darkness is inhibitory to floral induction in the long-day plant Hyoscyamus: darkness can be either *inhibitory* or *promotive* depending on the length of the cycle. Certainly any theory purporting to explain the phenomenon of photoperiodism must include an explanation of why the long-day plant Hyoscyamus flowers more readily when the duration of the light-dark cycle is increased above 24 hours by a simple extension of the dark period.

Finally, it has been established already that duration of the light-dark cycle controls the flowering response in the short-day plant Biloxi soybean. Now that the same has been found true for the long-day plant *Hyoscyamus niger*, it seems that the terms "long day" and "short day", while having practical significance for horticulturists, may have to be revised since they do not constitute accurate causal descriptions. However, it is premature to suggest a new terminology since data relating to periodicity in the flowering response in other long- and short-day plants are lacking.

Table III

Flowering Response of Hyoscyamus Niger Ann. to cycles of Several Durations; 2 Photoperiods (Expt III)

Duration of cycle, hr	Average days to appearance of elongation
Continuous light control:	10.4 ± 1.3
Series A: 10 hours' light each cycle:	
18	10.4 ± 1.4
24	No response
30	No response
36	27.6 ± 3.5
42	22.8 ± 2.4
48	23.1 ± 3.9
54	27.4 ± 1.8
60	30.2 ± 0.8*
Series B: 12 hours' light each cycle:	
18	9.1 ± 1.4
24	22.5 ± 2.4
30	No response
36	28.3 ± 3.7
42	21.1 ± 2.0
48	19.5 ± 1.6
54	24.1 ± 1.3
60	24.4 ± 0.8

Eight plants responded in each treatment, except as noted. Standard deviations shown.
* Six plants elongated; two died during treatment.

Summary

The flowering responses of the long-day-plant *Hyoscyamus niger* which resulted from exposure to several light-dark cycles of different durations revealed a very pronounced inhibition of flowering in plants exposed to cycles of 24 and 30 hours' duration. Cycles either shorter or longer resulted in much greater flowering. In fact, this long-day plant could be caused to flower on short photoperiods if each photoperiod was associated with either a long or a short dark period, while with intermediate dark periods flowering was inhibited. In other words, darkness can either promote or inhibit flowering in Hyoscyamus depending upon the duration of the light-dark cycle. The significance of this in relation to the rhythmic response of Biloxi soybean is discussed.

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Peter Boysen Jensen
1883–1959

Peter Boysen Jensen was born January 18, 1883, in South Jutland. Growing up on a farm, he early found himself enjoying the close contact with living nature which he loved and admired throughout his life. After one year of premedical studies, including botany, he made the decision, inspired by the ecologist Eugen Warming, to devote himself to plant physiology. Boysen Jensen's teachers and advisors in plant physiology were Rasmus Pedersen (1840–1905) and Wilhelm Johannsen (1857–1927). Neither of these was actively engaged in experimental plant physiology while Boysen Jensen was a student in Copenhagen. Johannsen was already pursuing his studies of the variation in populations and pure lines. Boysen Jensen received the degree of magister scientiarum in 1908. During the following year, he spent 3 months in the laboratory of Wilhelm Pfeffer in Leipzig and 4 months with the biochemist, E. Schulze in Zürich. Boysen Jensen's doctoral thesis (Copenhagen 1910) dealt with the breakdown of sugar during respiration in higher plants. Already in 1907 Boysen Jensen had been appointed scientific assistant in plant physiology at the University of Copenhagen. He gradually took over all teaching of plant physiology in the department and was recognized as lecturer in 1922. He succeeded Wilhelm Johannsen as professor in 1927.

Before Boysen Jensen there was no strong tradition for research in plant physiology in Denmark—although the foundation had been laid by Rasmus Pedersen and Johannsen. Boysen Jensen, on the other hand, succeeded in establishing plant physiology as a recognized and worthwhile experimental science in his country. This development, however, came slowly and as a result of many years of patient effort, for which Danish plant physiologists owe him an immeasurable debt.

Internationally, Boysen Jensen is known for his studies on the production of matter in photosynthesis and his work on plant growth substances. He realized that in order to analyze the process of production of matter, it was necessary to measure the rate of photosynthesis under conditions as close to natural as possible, primarily at normal CO_2-tension. He succeeded in constructing the necessary equipment, which became subsequently developed to a high degree of precision. His ideas and results are contained in "Die Stoffproduktion der Pflanzen" (Jena 1932) and in later publications by Boysen Jensen and others.

In 1910, Boysen Jensen published his first paper on the transmission of the phototropic stimulus in the oat coleoptile. He demonstrated beyond doubt that the transmission could take place through nonliving material, namely a thin layer of gelatin separating the unilaterally illuminated tip from the shaded stump. He was further able to show that the transmission took place in the part of the coleoptile, which was below the non-illuminated half of the tip, i.e., that longitudinal section which exhibits the greater rate of elongation during the phototropic curvature. These experiments were extended and published in greater detail in 1911 and 1913. In 1911, Boysen Jensen concluded from his experimental results that the transmission of the phototropic stimulus consisted in the transport "of a substance or of ions". These results were fundamental for the auxin theory of tropisms and thereby for the development of the auxin concept in general.

Boysen Jensen, however, did not immediately pursue a further study of the carrier of phototropic stimulus. He was actively engaged in investigations of the carbon cycle in the sea, and he also continued his studies of sugar metabolism in plants. Not until the 1920's were problems of tropisms and growth again made the object of investigation in his laboratory, first by students of Boysen Jensen, but later also by Boysen Jensen himself. Publications on growth substances alternated with publications on photosynthesis and production of matter. His book, "Die Wuchsstofftheorie und ihre Bedeutung für die Analyse des Wachstums und der Wachstumsbewegungen der Pflanze," came in 1935 and was later translated to English and Russian.

Among Boysen Jensen's other major literary achievements may be mentioned a textbook of plant physiology (two Danish and one German edition) and a broad treatise on biology, "Det levende" I–IV (1951–1953), in which, on the basis of a general survey of biology, he undertakes a discussion of the significance of biological concepts for sociology, ethics, theology, and other fields concerned with the life, behavior, beliefs, and ideals of man.

After his retirement at the age of 65 in 1948 Boysen Jensen continued his experimental work in his home and, in addition to "Det levende", published a dozen research papers, mostly on cellular determination and differentiation. His daughter, Margrethe was his devoted assistant in this work, which occupied Boysen Jensen until the day before his death on November 21, 1959.

Boysen Jensen was awarded honorary degrees from the Universities of Oslo, Norway, and Aarhus, Denmark. He was a member of the Royal Danish Academy, the American Academy of Arts and Sciences, and a number of academies in other countries. He was elected Corresponding Member of the American Society of Plant Physiologists in 1941 and of the Botanical Society of America in 1950. He became Honorary Member of the Danish Botanical Society in 1948.

His friends, colleagues, fellow plant physiologists, and students, of which a number came from the United States, will deplore the loss of this great thinker, gifted experimenter, and inspiring teacher.

—Poul Larsen

Walter Thomas
1880–1960

Dr. Walter Thomas, Professor Emeritus of Plant Nutrition at Pennsylvania State University, died at the age of 79 of a heart attack on February 1, 1960, in Miami, Florida. Through his long and distinguished career he was responsible more than any other man in this country, for the fundamental research work on the present widely accepted use of plant analysis in studying nutritional status and determining fertilizer requirements.

Walter Thomas was a native of Swansea, Wales, and graduated from the University of Wales in 1905. After spending a short time in Canada, he went to Pennsylvania State University in 1910 and remained there until his retirement in 1950 except for a two-year period during World War I when he was an officer in the Chemical Warfare Service of the British Army.

During his early years in Pennsylvania, he worked with various phases of soil analysis but concluded that these were not giving him the results he sought. He was encouraged by Dr. William Frear, Professor of Agricultural Chemistry, to study plant analysis to determine if chemical composition could be used as an index of plant yields. His earliest work clearly established that the composition of the leaf reflected added fertilizer treatments when yield responses were obtained. This was carried out with dwarfed apple trees grown in metal rims under different fertilizer treatments. A sidelight from this experiment was the discovery that all nitrate nitrogen was reduced in the fine roots of apple trees and thus no nitrates are present in apple leaves, a fact later confirmed by several workers.

In continuing work aimed at establishing the principles of plant analysis, Thomas became very interested in work being done in France by H. Lagatu and L. Maume on "Diagnostic Foliaire". He visited these workers in 1928 when in Europe receiving the degree of Doctor of Science from the University of Wales, and in succeeding years cooperated with them to establish more uniform procedures and means of expression. Some of the key features of present-day plant analytical procedures, including the selection of samples from morphologically homologous leaves and the importance of seasonal changes in nutrient-element composition were established during this period.

In 1937, upon transferring to the Department of Horticulture, his fine and fruitful association with the late Dr. Warren B. Mack commenced. Their work on foilar diagnosis dealt with the nutritional requirements of many crops and the relationship between nutrition and other factors, especially disease and environmental conditions. Their research primarily involved a study of fundamental relationships among nitrogen, phosphorus, and potassium and among the three bases, potassium, calcium, and magnesium. Through their concept that both intensity and balance had to be optimum for best nutrition, they established a very important principle which was first illustrated with corn in the historic Jordan fertility plots. Thomas and Mack were the first workers in the United States to emphasize the importance of balance between various nutrient-elements in interpreting the results of fertilizer experiments.

The work of Walter Thomas and his associates has proved to be very stimulating to many other plant physiologists and agricultural chemists and, while the exact procedures with which they worked are not widely used today, the validity and importance of most fundamentals they emphasized are receiving ever-increasing recognition. During most of Thomas' active years plant analysis was a very controversial subject. He showed courage in defending the principles he believed to be true.

Through his 78 publications Walter Thomas gained an international reputation resulting in a sizeable list of honors. Among these he was elected to the Court of Governors of the University of Wales in 1928 and was presented with the Charles Reid Barnes Honorary Life Membership award by the American Society of Plant Physiologists in 1943. In 1947 he was invited to read a paper on foliar diagnosis before the Twelfth International Congress of Pure and Applied Chemistry in London.

He gave his active support to forming the American Society of Plant Physiologists and served for many years on the finance committee where his extensive knowledge of the security markets proved most valuable especially during the depression years.

Walter Thomas was a true scholar. In his specialty, he had a very thorough knowledge of the whole field of plant analysis and could discuss in considerable detail the work being done by various investigators in all parts of the world, with many of whom he corresponded. After he retired at 70 his remarkably active and inquisitive mind continued to delve into many subjects. He could quote passages from Shakespeare, describe the composition of the latest missile fuel or discuss current stock market trends with equal authority and lively interest. He also kept up with his life-long hobbies of swimming and hiking, swimming at Miami Beach in the winter months and taking long hikes through the picturesque areas surrounding State College in the summer months.

Walter Thomas never married and was the last surviving member of his immediate family. His friends and those who are continuing and will continue the work he started will long remember the dedication and unselfishness that his life and work exemplified.—Cyril B. Smith.

News and Notes

Japanese Society of Plant Physiologists. The Japanese Society of Plant Physiologists (JSPP) was founded in April 1959. One of its chief activities will be to maintain a professional journal, Plant and Cell Physiology. The appearance of this new journal, which is issued quarterly, marks the fulfillment of a desire long cherished by plant physiologists in Japan. The primary aim of Plant and Cell Physiology is to acquaint the scientific public throughout the world primarily with work of Japanese scientists. However, foreign colleagues will be welcome as members of the JSPP and as contributors to the new journal. Membership and subscription fee is $5.00 per year. Correspondence concerning the society and the journal may be directed to the Editorial Offices of Plant and Cell Physiology, c/o Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Canadian Society of Plant Physiologists. The Canadian Society of Plant Physiologists now has a category of corresponding membership available at a nominal fee to plant physiologists who live outside Canada. Inquiries should be directed to Secretary-Treasurer, Dr. D. F. Forward, Department of Botany, University of Toronto, Toronto 5, Canada.

American Society of Plant Physiologists, *Midwest Section*: The sixth annual meeting of the Midwest Section was held at Michigan State University on June 20 and 21, 1960. Approximately 75 members were in attendance and several visitors were present. The program on Monday morning consisted of two concurrent sessions in which a total of 22 papers was presented. On the afternoon of the same day a "New Frontiers" symposium featured talks by Dr. B. Vennesland on "Photosynthetic Phosphorylation", Dr. Warren Butler on "Phytochrome, the Red-far-red Photochromic Pigment", and Dr. N. E. Tolbert on "A New Group of Plant Growth Substances".

Dr. R. H. Burris, section chairman, was master of ceremonies at the annual banquet in the evening. After the dinner, Dr. Georg A. Borgstrom presented a dynamic discussion of "World Food Problems".

On Tuesday morning, seven additional short papers were given in two concurrent sessions and this ended the meeting.

Officers elected for 1960 were: Dr. Alfred Sussman, chairman; Dr. J. B. Hanson, vice-chairman; and Dr. Sam Aronoff, representative to the American Society of Plant Physiologists (to complete the term of Dr. Harry Beevers who resigned to accept another office).—Carlos Miller, *Secretary-Treasurer, Midwest Section.*

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